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14. ABSTRACT

As a follow-up to contract DAMD17-94-V-4009, the inhalation toxicokinetics of sulfur mustard were studied in more detail in the hairless guinea pig as well as in the marmoset. Hairless guinea pigs were 5-min nose-only exposed to 0.3 and 1 LCt50. The distribution of sulfur mustard in the respiratory tract was measured at various time-points after exposure. The DNA-adduct levels increased and subsequently decreased with time. The highest levels were found in the upper airways. Marmosets were 5-min nose-only exposed to 160 $mg.m^{-3}$ sulfur mustard vapor in air, corresponding with 1 LCt50 in the hairless guinea pig. Sulfur mustard was easily measurable in blood during the absorption phase, reaching a maximum concentration of *ca.* 30 $ng.ml^{-1}$ at the end of the exposure period. The post-exposure concentration-time course could be described with a bi-exponential equation. The highest concentrations of intact sulfur mustard were found in bone marrow, liver and fat tissue, whereas the concentration in the lung was very low. DNA-adduct concentrations were much higher in the upper airways than in the tissues. Very low adduct levels were found in the lung and bone marrow. The results suggest that the marmoset is a better model for man with respect to the toxicology of inhaled sulfur mustard vapor than the hairless guinea pig.

15. SUBJECT TERMS
Sulfur mustard, inhalation, toxicokinetics, DNA-adducts, hemoglobin adducts, hairless guinea pig, marmoset

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I. INTRODUCTION

In spite of intensive research there is still no specific and causal therapy available for the local and systemic effects of intoxication with sulfur mustard, although promising results have been obtained very recently (*vide infra*). In general, treatment of casualties will have to be restricted to sustaining the vital functions of the patient, enhancing the healing process of the lesions and preventing secondary infections.
In order to provide a quantitative basis for pretreatment and therapy of intoxications with sulfur mustard the toxicokinetics of this agent as well as its major DNA-adduct (N7-HETE-gua) were studied for the intravenous, respiratory and percutaneous routes, within the framework of Cooperative Agreement DAMD17-94-V-4009 (Langenberg *et al.* 1998). The experiments were performed in the hairless guinea pig, which is considered to be a suitable animal model in studies of sulfur mustard since the thickness of the epidermis and the effect of sulfur mustard on the skin is reasonably comparable to that in man (Mershon *et al.* 1990).

For the analysis of intact sulfur mustard in blood and tissues we developed and validated a method using gas chromatography with mass-spectrometric detection, with a detection limit of 5 pg sulfur mustard/ml blood. The DNA-adducts of sulfur mustard in various relevant tissues were analyzed immunochemically, with a detection limit of 1 adduct per $4*10^8$ nucleotides. The combination of these analyses provides a unique insight into the relationship between the blood and tissue levels of intact sulfur mustard and the resulting DNA-damage.

The intravenous toxicokinetics of a dose of sulfur mustard corresponding with 1 LD50 (96-h, 8.2 mg/kg) and 0.3 LD50 in the hairless guinea pig were characterized by a very rapid distribution phase and a very slow elimination phase. The concentration of sulfur mustard in tissues (lung, spleen, liver, and bone marrow) exceeded that in blood shortly after i.v. administration of the agent, indicating extensive partitioning of sulfur mustard from blood into the tissues. A rapid DNA-adduct formation occurred in blood and lung, and subsequently in other tissues. Repair of adducts was considerable within 6 h. However, at 2 days after administration of sulfur mustard, adducts were still detectable in most of the tissues studied. Nonlinearity of the toxicokinetics with the dose was observed.

Next, the respiratory toxicokinetics were studied. Rather surprisingly, the intact agent could not be detected in blood during and after 5-min exposure of animals to 1 LCt50 of sulfur mustard (96-h, 800 $mg.min.m^{-3}$). Low concentrations of N7-HETE-gua were detectable in blood and lung tissue. However, sulfur mustard was measurable in the blood upon an 8-min nose-only exposure of hairless guinea pigs to 3 LCt50 of the toxicant at concentrations up to 1.8 ng/ml. Toxicokinetic evaluation of the concentration-time profile was only possible by assuming a very rapid absorption process in combination with a slow absorption process. In this case, measurable concentrations of N7-HETE-gua in DNA were found in blood, lung tissue, and spleen.

The respiratory tract was isolated from animals that were nose-only exposed to 1 LCt50 of sulfur mustard in 5 min, at 4 h after ending the exposure. The tract was divided into 6 regions. Most adduct formation had occurred in the larynx and trachea, whereas hardly any N7-HETE-gua was found in the lung. Thus, the observation that sulfur mustard was not detected in the blood of animals exposed to 1 LCt50 of sulfur mustard may be explained from the almost exclusive distribution of the agent within the respiratory tract. Evidently, respiratory exposure to sulfur mustard leads predominantly to local damage in the respiratory tract, at least in (hairless) guinea pigs.

The percutaneous toxicokinetics of sulfur mustard in the hairless guinea pig were also studied. During and after a *ca.* 45-min percutaneous exposure of the animals to a Ct of 10,000 $mg.min.m^{-3}$, concentrations of sulfur mustard in blood up to *ca.* 12 ng/ml could be detected. As in the case of nose-only exposure to a Ct of 2,400 $mg.min.m^{-3}$, the existence of a rapid and a simultaneous slow absorption process was suspected. Concentrations of intact sulfur mustard exceeding those in blood were measured in the tissues shortly after ending the exposure. Rather low concentrations of N7-HETE-gua were measured in DNA of most tissues. We

concluded tentatively that systemic intoxication from sulfur mustard is more likely to occur from a percutaneous exposure than from a respiratory exposure, at least in the hairless guinea pig.

Based on the amount of DNA-adducts in the skin, Topical Skin Protectants 1511 and 2701 (obtained from the U.S. Army Medical Research Institute of Chemical Defense) protected hairless guinea pigs almost completely against skin damage caused by sulfur mustard vapor. TSP 2701 performed somewhat better against liquid sulfur mustard than TSP 1511.

Results of our investigations on diagnosis/retrospective detection of exposure to sulfur mustard, within the context of Cooperative Agreement DAMD17-97-2-7002 (Benschop *et al.* 2000) showed, surprisingly, that adduct levels of sulfur mustard to hemoglobin in guinea pigs and marmosets increase for several days after intravenous bolus administration of the toxicant. Subsequently, the level of hemoglobin adducts decreases, still being detectable at 56 and 92 days after sulfur mustard administration in guinea pigs and marmosets, respectively. Therefore, protein-adduct formation can be regarded as cumulative on a relevant time scale. This result stands in sharp contrast with the concentration-time profile of DNA-adducts, due to repair and/or removal of such adducts.
At least two aspects of this result should be taken into consideration. Firstly, it can have consequences for treatment of systemic effects of intoxication with sulfur mustard, since hemoglobin-adduct formation will probably be parallel with adduct formation to other proteins which play a role in the etiology of damage due to exposure to sulfur mustard (Mol, 2000). For example, it may become relevant to attempt removal of sulfur mustard from circulation. Secondly, the late formation of hemoglobin-adducts can be used as a measure for the time period in which toxicologically relevant levels of sulfur mustard are present in the body. The definition of this time period has been elusive so far and may now be investigated on the basis of 'post-exposure hemoglobin-adduct formation'.

In summary, our toxicokinetic studies of sulfur mustard as performed within the context of the abovementioned Cooperative Agreements have provided elucidating data on the fate of this agent in hairless guinea pigs, especially with regard to the relative importance of local and systemic effects upon respiratory and percutaneous exposure.

It seems difficult to reconcile our conclusion that respiratory exposure leads predominantly to local damage with the results of our measurements of N7-HETE-gua in DNA from blood of Iranian casualties of sulfur mustard exposure (Benschop *et al.* 1997). As judged from their overall condition, these patients were exposed to rather small amounts of sulfur mustard vapor via the respiratory route, since skin damage was hardly noticable. Nevertheless, in blood samples taken at 3 to 4 weeks after exposure we found blood levels of N7-HETE-gua in DNA of these patients corresponding with those in hairless guinea pigs immediately after nose-only exposure to up to 3 LCt50 of sulfur mustard vapor. Furthermore, we measured hemoglobin-adduct levels corresponding with those found in human blood after *in vitro* treatment with 0.9 μM sulfur mustard. These contrasting results suggest that species differences may play a major role in determining the relative importance of local and systemic damage upon respiratory exposure.

Shortly, we proposed to address items as indicated above in the current contract:

(i) Comparative inhalation toxicokinetics of sulfur mustard and N7-HETE-gua in the hairless guinea pig and in a species more relevant for man, i.e., the marmoset;

(ii) The percutaneous toxicokinetics of sulfur mustard and N7-HETE-gua in the hairless guinea pig at a dose comparable with that of the respiratory exposure as investigated in the previous contract;

(iii) The time period during which toxicologically relevant levels of sulfur mustard are present in marmosets, as based on late formation of stable hemoglobin-adducts;

Unfortunately, due to budgetary restrictions the work programme of the contract had to be revised in December 2004, upon which items (ii) and (iii) were skipped.

In the following item (i) is further elucidated.

Initially, we were rather surprised that the concentrations of sulfur mustard in blood were below the detection limit during and after nose-only exposure of hairless guinea pigs to 1 LCt50. However, the observation that N7-HETE-gua could not be detected in spleen, bone marrow and small intestine, while rather low concentrations of N7-HETE-gua were measured in the lung at 10 min and 48 h after ending the exposure is in agreement with this outcome. Most clarifying was the distribution of N7-HETE-gua within the respiratory tract at 4 h after ending the 5-min nose-only exposure. Adduct formation occurred mainly in the upper airways, whereas almost no sulfur mustard appeared to have reached the lung.
From these findings it was concluded that sulfur mustard apparently causes lethal damage in the respiratory tract without a systemic uptake to such an extent that systemically lethal concentrations can build up. This mechanism stands in contrast with that for nerve agents, which are absorbed in the (upper) respiratory tract to cause mainly systemic toxicity.
Cameron *et al.* (1946) have also observed that hardly any sulfur mustard reaches the lung in rabbits, based on measurements of the concentration of sulfur mustard in air sampled from the trachea via a cannula. They hypothesized that absorption of lethal doses of sulfur mustard occurs via the nasal mucosa. However, since we could not detect sulfur mustard in blood the present results seem to suggest that the respiratory toxicity of sulfur mustard is overwhelmingly of a local rather than of a systemic nature, at least in the (hairless) guinea pig.
Within the context of Cooperative Agreement DAMD17-92-V-2005, Benschop and Van der Schans (1995) established exposure to sulfur mustard of two Iranian victims of the Iran-Iraq conflict in 1988 by measurement of DNA-adducts in lymphocytes and granulocytes as well as adducts to hemoglobin (Benschop *et al.*, 1997). The blood samples in which the exposure was verified were taken at 22 and 26 days after the alleged exposure. One victim suffered from injuries compatible with sulfur mustard intoxication but did not have lung injuries; the symptoms of the other victim were only vaguely compatible with sulfur mustard intoxication. Interestingly, the N7-HETE-gua levels and adduct levels to hemoglobin measured in these Iranian victims are comparable with those measured in hairless guinea pigs immediately after nose-only exposure to 0.3-3 LCt50. As deduced from the clinical signs, the Iranian victims appeared <u>not</u> to be severely intoxicated, whereas the hairless guinea pigs were. Furthermore, one has to take into account that a long period of time had passed between exposure of the Iranian victims and collection of the blood samples. Since DNA-adducts will disappear with time due to repair mechanisms and/or removal of the damaged white blood cells, there seems to be a large discrepancy between adduct formation resulting from respiratory exposure between humans and guinea pigs.
A tentative explanation for this discrepancy is the high complexity of the nasal region of the guinea pig (Schreiber and Raabe 1981). Obviously, in species with a less complex nasal system such as man, a larger fraction of the inhaled sulfur mustard may reach the lung. This may lead to lung damage by direct reaction of sulfur mustard with the lung tissues and/or indirectly via systemic uptake. We proposed to study the inhalation toxicokinetics of sulfur mustard in the marmoset monkey, of which the anatomy of the naso-pharyngeal airway is much more similar to that in man, in order to test this hypothesis. The marmosets will be nose-only exposed to a Ct of 800 mg.min.m^{-3} in 5 min, which corresponds with 1 LCt50 (96-h) in the hairless guinea pig. The concentration-time course of sulfur mustard in blood will be determined, and the concentrations of intact sulfur mustard and N7-HETE-gua in blood and tissues will be measured at various time points after ending the nose-only exposure, as well as the concentrations of the sulfur mustard adducts to hemoglobin.
Furthermore, the distribution of N7-HETE-gua within the respiratory tract of the marmoset will be studied, as was previously performed for hairless guinea pigs (Langenberg *et al.* 1998).
In addition, the time course of the distribution of N7-HETE-gua within the respiratory tract of the hairless guinea pig will be studied, by measurement of the adduct at various time points after ending the nose-only exposure to 1 and 0.3 LCt50.

As stated above, very recently promising results have been obtained in the search for a causal antidote for sulfur mustard. Within the context of Cooperative Agreement DAMD17-02-1-0206 Dr. Marijke Mol from our laboratory is studying the mechanism of action of sulfur mustard using proteomics, which has led to new hypotheses concerning how the toxic effects of sulfur mustard come to be. Based on her hypothesis that matrix metalloproteases are involved in the blister formation process, she has tested various types of inhibitors of these proteases. Of these the compounds Ilomastat (QuickMed Technologies, USA) and BB94 (British Biotechnologies, UK) appeared to be highly effective in preventing blister formation on human skin exposed to saturated sulfur mustard vapor, also when applied several hours after exposure to the vesicant, at least *in vitro* (Mol 2004). So there is justified hope that a causal antidote for sulfur mustard will become available in the near future.

II. EXPERIMENTAL PROCEDURES

II.1. Materials

WARNING:Sulfur mustard is a primary carcinogenic, vesicating, and cytotoxic agent. This compound should be handled only in fume hoods by experienced personnel.

Technical grade sulfur mustard was purified by fractional distillation in a cracking tube column (Fischer, Meckenheim, Germany) to a gas chromatographic purity exceeding 99.5 %. The internal standard, D8-sulfur mustard was obtained as described elsewhere (Benschop and Van der Schans, 1995). Ethyl acetate ('zur Rückstandsanalyse') was procured from Merck (Darmstadt, Germany) and was distilled over a column packed with Dixon rings (plate number 80; NGW, Wertheim, Germany) before use. Isopropanol (purity > 99.5 %) was purchased from Fluka (Buchs, Switzerland).
The following products were obtained commercially and were used without further purification: heparin (Vitrum, Stockholm, 5000 IU/ml), ketamine hydrochloride (Vetalar®, Parke-Davis, Morris Plains, NJ, USA), buprenorfine hydrochloride (Temgesic®, Schering-Plough, Amstelveen, The Netherlands), HPLC-grade water (Fisons, Loughborough, UK), Tenax TA, 60-80 mesh (Chrompack, Middelburg, The Netherlands). Disodium edetate (EDTA), Triton® X-100, sodium chloride, potassium chloride, disodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate, phenol, phosphoric acid (85 %), chloroform and ethanol were obtained from Merck (Darmstadt, Germany); sodium dodecyl sulfate (SDS), Tris.HCl from J.T. Baker (Phillipsburg, NJ, USA), and isoamyl alcohol from UCB (Brussels, Belgium); calf thymus DNA, proteinase K (EC 3.4.21.14, activity ca. 20 units/mg protein) and RNase T1 (EC3.1.27.5, activity ca. 40 units/mg protein) from Boehringer (Mannheim, Germany); RNase A, and Tween 20 from Sigma Chemical Co. (St. Louis, MO, USA); and skimmed milk powder, less than 1 % fat, from Campina (Eindhoven, The Netherlands).

Immunoslotblot assays were carried out with Schleicher & Schuell minifold S (6 mm^2 slots; Schleicher & Schuell, Dassel, Germany) and nitrocellulose filters (pore size 0.1 µm; Schleicher and Schuell). DNA was immobilized by UV-crosslinking with a GS Gene Linker UV chamber (Bio-Rad Laboratories, Hercules, CA, USA). The enhanced chemiluminescence blotting detection system (Boehringer) was used for the detection of peroxidase activity. The developed film was scanned with a densitometer (Ultroscan XL, Pharmacia). In later experiments the chemiluminescence was recorded with a 1450 MicroBeta Trilux Luminescence Counter (EG & G Wallac, Breda, The Netherlands).

II.2 Determination of N7-HETE-gua in DNA

DNA isolation
Mucosal epithelia from different locations in the respiratory tract of the hairless guinea pig and marmoset; nasal cavity, nasopharynx, larynx, trachea and carina, were collected by scraping off the mucosal surfaces. Lung tissue was homogenized prior to DNA isolation.
For DNA isolation all samples were incubated overnight in 300 µl cell lysis solution supplemented with Proteinase K (100µg/ml), under continuous slow shaking at room temperature. Then, RNAse A treatment (50 µg/ml) was carried out for 15 min at 37 °C, followed by cooling on ice for 5 min. To precipitate cellular debris 100 µl Protein Precipitation Solution was added and incubated on ice for 5 min. After centrifugation at 5,200g for 10 min the supernatant was transferred to a new vial containing 300 µl isopropanol to precipitate the DNA. After centrifugation at 5,200g for 5 min the pellet was washed with 70% ethanol, centrifuged at 5,200g for 5 min and dried on air. The DNA was dissolved in 50 µl 0.1TE buffer under continuous shaking at room temperature.

DNA denaturation
Double-stranded calf thymus DNA and DNA samples isolated from respiratory organs from the hairless guinea pig and marmoset after sulfur mustard vapor exposure were denatured to single-stranded DNA in 0.1 TE buffer containing 4.1% formamide and 0.1% formaldehyde (50 µg DNA/ml) at 52 °C for 15 min, followed by rapid cooling on ice and storage at -20 °C. It is essential that, after denaturation, the DNA samples were frozen at least one time before application in the immunoslotblot assay.

Immunoslotblot procedure for N7-HETE-Gua
Two-hundred µl single-stranded DNA (5 µg/ml) containing N7-HETE-Gua was slot blotted onto a nitrocellulose filter. As a reference, calibration samples of calf thymus DNA with adduct levels in the range of 0-10 N7-HETE-Gua/10^7 nucleotides were included. All samples were blotted in duplicate on the same filter. After rinsing the slots with PBS the filters were air dried. Subsequently, DNA was immobilized by UV crosslinking (50 mJ.cm^{-2}).
The next steps in the procedure, i.e., treatment with blocking solution, 1st antibody (2F8, directed against N7-HETE-Gua in DNA) and 2nd antibody (rabbit-anti-mouse-Ig-horse radish peroxidase), were performed as previously described (Benschop and Van der Schans, 1995). Solutions A and B of the chemiluminescence blotting detection system were mixed (100:1) and equilibrated for 1 h at 25 °C. The filters were incubated for 1 min in substrate solution and placed in a plastic bag. Excessive substrate was pressed out. Next, the filters were placed in a luminometer and the chemiluminescence was measured. All experiments were performed at least in duplicate.

The Standard Operating Procedure for determination of sulfur mustard adducts to DNA in respiratory tract of the hairless guinea pig is described in Annex B.

II.3. Gas chromatography of sulfur mustard
Configuration 1: TDSA-GC-MSD
Sulfur mustard in biological samples was determined by gas chromatography with mass-selective detection and large volume injection.
The GC-MS configuration consisted of an Agilent 6890A GC, equipped with a Gerstel Thermo Desorption Autosampler device (TDSA) and an Agilent 5973 Mass Selective Detector (MSD) (Agilent Technol. Inc., Wilmington, DE, USA).
The GC was fitted with a 30 m x 0.25 mm I.D. VF-5MS fused silica column with a film thickness of 1.00 µm (Chrompack Varian), inserted directly into the mass spectrometer source.
The desorption tubes for the TDSA were filled with 300 mg Tenax TA (60-80 mesh) and a loosely packed plug of dimethyldichlorosilane-treated glass wool of about 3 to 4 cm to promote evaporation of the large volume of the solvent. Before use, the Tenax tubes were preconditioned by heating under a stream of helium or nitrogen at 220°C for 8 h.
Tenax tubes were loaded with 100 to 400 µL of the sample extracts in portions of 100 µL using a Hamilton 100 µL syringe with a loading interval time of 5 min. During this step, a gentle stream of nitrogen (250 mL/min) flowed through the tubes to evaporate the solvent. Fifteen min after the final loading step, the Tenax tube was installed into the TDSA. The TDSA oven temperature was started at 30 °C and then ramped at 60 °C/min to 220 °C where it was held for 5 min. During desorption, the helium flow rate through the tube was 30 mL/min. The TDSA-GC transfer line was set at 220°C.
The desorbed sample components were trapped in splitless mode by a CIS which was cooled to –50°C with liquid nitrogen. After desorption, the trapped components were transferred in splitless mode to the capillary column by rapid heating of the CIS at 12°C/s to 220°C where it was held for 5 min. Splitless time was 1 min and purge flow was 50 mL/min. The initial GC oven temperature was 65°C for 4 min, then ramped at 20°C/min to 260°C and held at this temperature for 11 min. The helium column flow during analysis was set at 1 mL/min in constant flow mode. The GC-MSD transfer line was heated at 220°C. The MSD was operated in the selected ion mode (SIM) using electron impact (EI) ionization (70 eV) and the ions monitored were m/z

109 for sulfur mustard and 115 for D8-sulfur mustard, with a SIM dwell time of 80 ms. Temperature of the quadrupole was maintained at 150°C, temperature of the ionization source was 230°C.

Configuration 2: TDSA-MUSIC-GC-MSD
This configuration was obtained by inserting a Multiple Switching Intelligent Controller (MuSIC, Chrompack/Varian, Middelburg, The Netherlands) in configuration 1, to enable two-dimensional chromatography.
The ethyl acetate extract (200-400 µl) was loaded onto a tenax tube with *ca.* 300 mg tenax TA 60-80 mesh and flushed dry with nitrogen (flow 250 ml/min) during 30-45 min. Next, the components were desorbed during 6 min at 220°C and trapped in a Gerstel CIS 4 injector liner at -50°C. After the desorption step, the sample components are splitless injected at 250 °C (1 min, 50 ml/min) onto the CP-Sil 19CB Varian pre-column (50 m * 0.32 mm i.d., d_f 0.20 µm) with a constant helium carrier gas pressure of 100 kPa. The oven temperature of the Agilent 6890 GC was programmed from 70 (2 min constant) to 223°C at a rate of 10 °C/min. The mustard gas fraction is trapped from 16.3-17.4 min at -70°C and re-injected on the CP VF-5 MS Varian analytical column (30 m * 0.25 mm i.d., d_f 1 µm) when the oven is cooled down to 80°C. After waiting for 2 min, the temperature is programmed to 170°C at a rate of 5°C/min. Next, the temperature is programmed to 250°C during 5 minutes to elute high-boiling compounds. The MuSIC pressure for the analytical column is set at 54 kPa. Chemstation is used for data acquisition.
The analytical column is connected to an Agilent 5973 MSD, which operates in the EI mode. Detection at PMT voltage of 1500 V and 70 eV takes place at *m/z* 109-111 and 115-117 for sulfur mustard and D8-sulfur mustard respectively (dwell time 80 msec). The source temperature is set at 230°C, transfer line at 220°C and the quad at 150°C.

II.4. Gas chromatographic analysis of the sulfur mustard adduct to the terminal N-valine of hemoglobin
The sample preparation of the adduct of sulfur mustard to the terminal N-valine of hemoglobin (HETE-val) in marmoset blood is described in detail in Noort *et al.* (2004). Shortly, globin is isolated from blood, and subjected to the modified Edman degradation, after which the desired product is obtained by reaction with heptafluorobutyric imidazole.
GC-MS analysis was performed on a HP 5973 mass selective detector which was connected to an Agilent 6890 GC system with a PAL autosampler, using splitless injection (270°C, 1 min splitless time, flow 50 ml/min) on a UNIS PTV injector with a large volume liner. The system was operated in the NICI mode (methane, flow setting 40%) with a transfer temperature of 240°C, a source temperature of 230°C, a quad temperature of 150°C and an ionization energy of 70 eV. The column used was a CPSil 8 CB fused-silica capillary column (25-m length, 0.25-mm i.d., 1.2-µm film thickness, Varian) or a DB-35MS fused-silica capillary column (30-m length, 0.25-mm i.d., 0.25-µm film thickness, Restek). The column flow was set at 3 and 1 ml/min respectively (helium) constant flow mode. The oven of the chromatograph was kept at 100°C for 1 min; the temperature was then programmed to 280°C at 20°C/min and subsequently held at this temperature for 30 min. Injection volume was 3 µl (containing about 3% of the total sample).
Ion chromatograms were recorded after monitoring for *m/z* 564 (M-.-3 HF, analyte), 572 (M-.-3 HF, internal standard and 157 (dwell time 80 msec). Because of interfering peaks at *m/z* 654 and 572, calculations were based at peakheight ratio of m/z 157 of the d0/d8-N-HETE-VAL-PFPTH-HFB. A 0.5 µM d8-HETE-VAL internal standard was present in the samples and on this base the concentration was calculated. It seemed that human plasma was cleaner than marmoset plasma, and for that reason not all ions could be used for identification (a control sample from human plasma was analysed and could be used for all ions). For this reason the detection limit was 0.1-0.2 µM based on the ion *m/z* 157.

II.5 Vapor exposure of animals

Apparatus for generation of sulfur mustard vapor
The apparatus for controlled generation of SM vapor in air is described in detail elsewhere (Langenberg *et al.* 1998).

Apparatus for nose-only exposure of hairless guinea pigs to sulfur mustard vapor
The exposure apparatus is also decribed in detail in Langenberg *et al.* (1998).

Apparatus for nose-only exposure of marmosets to sulfur mustard vapor
Development of such an apparatus is one of the Technical Objectives of this Cooperative Agreement, and is described in paragraph III.3.

II.6 Animal experiments

Animals
Male hairless guinea pigs [300-400 g; species identification Crl:IAF(HA)BR] were purchased from Charles River USA (Wilmington, MA, USA). The animals were allowed to eat and drink *ad libitum*. They were allowed to acclimatize to their new environment for at least 1 week before they were used in any experiment. Two Standard Operating Procedures are applicable to the housing and care of the hairless guinea pigs, i.e., 'Ordering and Housing of Experimental Animals' (SOP Q213-W-039) and 'Cleaning and Maintenance of Animal Facilities' (SOP Q213-W-040).
Male and female marmoset monkeys [*Callithrix jacchus*, 150-250 g], were obtained from the Biomedical Primate Research Center (Rijswijk, The Netherlands) which is located next to TNO-PML. Three animals were purchased from the Deutsche Primaten Zentrum (Goettingen, Germany). In accordance with SOP Q215-W-058 the animals were housed individually in stainless steel wire cages at a temperature (25 °C) and humidity (50 %) controlled room. They were allowed to drink *ad libitum*. Food supply was according to the aforementioned SOP.
The protocols for the animal experiments were approved by the TNO Committee on Animal Care and Use; under registration numbers DEC 1513 (hairless guinea pig experiments) and DEC 1514 (marmoset experiments).

Nose-only exposure of hairless guinea pigs to sulfur mustard vapor
Hairless guinea pigs were weighed and subsequently anesthetized with a combination of racemic ketamine hydrochloride (Vetalar®, 50-100 mg/kg, i.m.) and acetylpromazine maleate (Vetranquil®, 0.05-0.1 mg/kg, i.m.). Next, they were immobilized in the modified Battelle-tube (see Langenberg *et al.* 1998) and nose-only exposed to sulfur mustard vapor in air for 5 min. At various time points after ending exposure (10 and 30 min, 1, 2, 4 and 24 h) groups of anesthetized animals (n=4 per time point) were euthanized by intraperitoneal administration of an overdose (0.4 ml) of sodium pentobarbital (Nembutal®),after which the respiratory tract was isolated for analysis.

Inhalation toxicokinetics of sulfur mustard and adducts in the marmoset
Marmosets were weighed and subsequently anesthetized with 20 mg of racemic ketamine hydrochloride (Nimatek®, i.m.) and acepromazine maleate (Vetranquil®, 50 µl of 10 mg.l^{-1}, i.m.). Next, a cannula was inserted into the femoral artery, heparine was administered via the cannula (60 U) and the animal was fixated in a specially designed chair (see paragraph III.3). A blood sample was drawn via the cannula (time zero) and an equal volume of sterile saline was given back to the animal, via the same cannula. The mouthpiece of the fixation chair was connected to the sulfur mustard vapor generation apparatus and the animal was nose-only exposed to 160 mg.m^{-3} of sulfur mustard vapor in air for 5 min. Blood samples were drawn at 1, 3 and 5 min during the exposure and various time points thereafter. Each drawn volume of blood was replaced with sterile

saline. At 60 min after starting the exposure, the anesthetized animal was euthanized by exsanguination. The animal was dissected rapidly and organs/tissues of interest (liver, spleen, bone marrow, small intestine, fat) were collected. Furthermore, samples were taken from mucosa in the respiratory tract (nasal mucosa, nasopharynx, larynx, trachea, carina, and lung).
In separate experiments marmosets were weighed and anesthetized and subsequnetly nose-only exposed to 160 $mg.m^{-3}$ of sulfur mustard in air for 5 min. The animals were euthanized with an overdose of Nembutal® (0.4 ml, i.p.) at various time points: 10 and 30 min and 24 h after the start of the exposure. Next, the animals were dissected rapidly and organs/tissues of interest (lung, liver, spleen, bone marrow, small intestine, fat) were collected.

II.7 Curve-fitting of toxicokinetic data

Curve-fitting of the measured concentration-time course was performed by nonlinear regression with the Microsoft Windows version of TableCurve (Jandell, AISN Software) on a personal computer equipped with a Pentium IV processor.
The data were fitted to a discontinuous function:

$[agent]_t = A + B*t$, for the exposure phase($t = 0$-5 min)

$[agent]_t = C*e^{-ct} + D*e^{-dt}$, for the post-exposure phase ($t > 5$ min)

by calculation of the parameters A, B, C, D, c and d. In these equations, $[agent]_t$ is the concentration in blood of nerve agent (stereoisomer) at time t. After the determination of the adequate curve-fit, several toxicokinetic parameters were calculated:

area under the curve (AUC)	AUC = C/c + D/d + .[agent] at time 5 min*5/2	
half-life of distribution ($t_{½,dis}$)	$t_{½,dis} = \ln 2/\alpha$	(eq. 12)
terminal half-life ($t_{½,el}$)	$t_{½,el} = \ln 2/\beta$	(eq. 13)

III. RESULTS AND DISCUSSION

III.1 TIME COURSE OF N7-HETE-GUA IN THE RESPIRATORY TRACT OF HAIRLESS GUINEA PIGS AFTER 5-MIN NOSE-ONLY EXPOSURE TO 1 LCt50 SULFUR MUSTARD IN AIR (T.O. 1)

Animals were weighed, anesthetized and 5-min nose-only exposed to sulfur mustard vapor in air, at a concentration of 160 $mg.m^{-3}$. This results in an exposure dose of 800 $mg.min.m^{-3}$, which corresponds with 1 LCt50 (96-h), as determined within the context of Cooperative Agreement DAMD17-94-V-4009 (Langenberg *et al.* 1998). Animals were euthanized at various time points, i.e., at 10 and 30 min, and at 1, 2 and 24 h after ending the 5-min nose-only exposure. The 4-h time point was measured previously (Langenberg *et al.* 1998). For each time point n=4. Samples from the mucosa of various sites of the respiratory tract, i.e., from the nasal cavity, nasopharynx, larynx, trachea and carina, were collected by scraping off the mucosal surfaces. Furthermore, the lungs were sampled. In all of these samples the amount of adducts of sulfur mustard to guanine in the DNA (N7-HETE-gua) was determined.

The vapor of sulfur mustard in air was generated, monitored semi-continuously on-line, and fed into the exposure apparatus using the equipment developed within the context of the abovementioned Cooperative Agreement. In short, a number of mass-flow controllers in combination with specially-designed glass tubings and a storage vessel for the neat sulfur mustard were used to control and influence the vapor concentration. Several valves and pressure gauges were installed for safety reasons. Where possible, the equipment was thermostatted. The exposure module was equipped with a three-way valve in combination with pressure monitoring, activated carbon canisters and needle valves to allow the operator to handle the equipment in a safe way.
The concentration of the sulfur mustard vapor in air was monitored semi-continuously by means of a gas chromatograph equipped with a fixed volume gas sampling valve, a capillary column (BP-1, length *ca.* 8 m and an internal diameter of 0.32 mm with a film thickness of 1 μm) and an FID. For calibration purposes this column is also connected to an on-column injector by means of a glass press-fit connector and an uncoated fused silica column (*ca.* 1 m length and 0.32 mm i.d.). This allows the operator to calibrate the complete system without disconnecting the column from the gas sampling valve.
Standard solutions of sulfur mustard in ethyl acetate were prepared for the calibration. Data-acquisition was performed with a combination of an amplifier (constructed in-house at TNO-PML), a National Instruments Daq-board installed in a Windows™ PC and a 'home-made' written software program for analyzing the data. In Table 1 the individual averaged vapor concentrations as well as the deviation of these concentrations are presented.

Distribution of N7-HETE-Gua within the respiratory tract of the hairless guinea pig after 5-min nose-only exposure to sulfur mustard vapor in air of 1 LCt50.
The concentration of N7-HETE Gua was measured in the respiratory tract samples with the immuno-slot-blot procedure described in paragraph II.2 and Annex B. Immunodetection of N7-HETE-Gua has proven to be a sensitive, reproducible and reliable method to quantify damage caused by sulfur mustard (Benschop *et al.* 1997, van der Schans *et al.* 1996, Langenberg *et al.*, 1998).
The concentrations of N7-HETE-gua measured in the respiratory tract samples of the individual animals as well as the averaged values with SEM per group of 4 animals for each time point are presented in Tables 2 and 3, respectively. The distribution of N7-HETE-Gua within the respiratory tract at the various time points after exposure is presented in Figure 1.

Table 1. The mean concentration (± S.D., $mg.m^{-3}$) of sulfur mustard vapor in air to which the individual animals were nose-only exposed.

Guinea pig number	Weight (g)	Concentration ($mg.m^{-3}$)	S.D. ($mg.m^{-3}$)	Sampling time (min)
HD 029	598	171.8	8.0	10
HD 030	592	171.5	6.6	10
HD 031	714	162.4	8.0	10
HD 032	696	158.9	8.9	10
Average	**650**	**166.2**		
HD 033	597	156.5	12.3	30
HD 034	581	171.0	9.5	30
HD 035	619	161.7	7.7	30
HD 036	630	162.7	6.2	30
Average	**607**	**163.0**		
HD 037	624	169.0	9.8	60
HD 038	545	171.9	4.7	60
HD 039	709	160.0	11.5	60
HD 040	726	167.8	11.9	60
Average	**651**	**167.2**		
HD 041	581	167.3	8.2	120
HD 042	523	165.2	9.0	120
HD 043	666	162.3	7.8	120
HD 044	586	168.8	16.2	120
Average	**589**	**165.9**		
HD 045	611	163.7	7.7	24 h
HD 046	546	171.8	6.6	24 h
HD 047	746	161.5	13.5	24 h
HD 048	643	154.9	19.4	24 h
Average	**637**	**163.0**		
Average	**627**	**165.0**	**5.3**	

N7-HETE-Gua could be detected in the nasal cavity, nasopharynx, larynx, trachea, carina, and in lung tissue following 5-min nose-only exposure to 1 LCt50 of sulfur mustard vapor in air. The highest adduct levels were found at 10-120 min after the start of the exposure in the nasopharynx, larynx, trachea and carina (up to *ca.* 45 adducts per 10^7 nucleotides). The lowest adduct levels were found in lung tissue, up to *ca.* 8 adducts per 10^7 nucleotides.

In general the adduct levels appear to build up relatively rapidly after nose-only exposure to this concentration of sulfur mustard vapor in air and decrease quite rapidly within the first 24 h after ending the exposure.

Table 2. N7-HETE-gua (expressed as number per 10^7 nucleotides) in respiratory tract regions of individual hairless guinea pigs at various time points after 5-min nose-only exposure to 160 $mg.m^{-3}$ of sulfur mustard vapor in air, corresponding with 1 LCt50.

	10 min	*30 min*	*60 min*	*120 min*	*240 min*	*24 h*	*control*

	HD031	HD035	HD039	HD043	GS1[a]	HD047[b]	HD025
Nasal mucosa	75	7.2	3.3	70	30	f.a.	0
Nasopharynx	72	83	71	76	57	f.a.	0
Larynx	68	83	81	70	89	f.a.	0
Trachea	77	82	64	66	91	f.a.	0
Carina	73	80	80	67	f.a.	f.a.	0
Lung	1.4	1.5	2.6	14	0.4	f.a.	0

	HD032	HD036	HD040	HD044	GS2[a]	HD048	HD026
Nasal mucosa	59	16	54	58	57	0.65	0
Nasopharynx	60	15	61	57	f.a.	6.5	0
Larynx	55	61	64	57	85	41	0
Trachea	56	59	57	57	93	25	0
Carina	59	57	54	51	57	4.0	0
Lung	12	3.3	1.0	15	0.09	0	0

	HD029	HD033	HD037	HD041	GS3[a]	HD045	HD027
Nasal mucosa	16	22	11	0.15	86	1.8	0
Nasopharynx	15	2.9	14	42	90	3.6	
Larynx	13	26	25	47	92	9.2	0
Trachea	49	21	3.9	47	86	23	0
Carina	24	n.d.	22	n.m.	64	2. 8	
Lung	0.19	0.76	0.15	0.17	0.6	0.09	0

	HD030	HD034	HD038	HD042	GS4[a]	HD045	HD028
Nasal mucosa	0.57	20	0.08	2.4	49	12	0
Nasopharynx	14	0.13	4	14	61	3.2	0
Larynx	30.18	21	28	13	88	6.9	0
Trachea	29	25	11	5.6	90	2.0	0
Carina	9.7	15	26	5.8	36	1.2	0
Lung	0.23	0.83	0.55	0.54	0.3	0.09	0

[a] Data from DAMD17-94-V-4009 (Langenberg *et al.* 1998)
[b] Animal HD047 died overnight, sampling respiratory tract useless
f.a. = failed analysis
n.m. = not measured

The adduct levels measured in the previous study (Langenberg *et al.* 1998) in hairless guinea pigs at 240 min after 5-min nose-only exposure to 1 LCt50 sulfur mustard vapor in air appear somewhat too high to adequately fit in with the concentration-time course of the DNA-adducts as measured in the current study. In view of the fact that these data are about 8 years old and measured in animals obtained from a different source (and continent) the apparent discrepancy is quite reasonable.

Table 3. Mean concentrations of N7-HETE-gua (expressed as number per 10^7 nucleotides) with SEM (n=4, unless stated otherwise) in respiratory tract regions of individual hairless guinea pigs at various time points after 5-min nose-only exposure to 160 mg.m^{-3} of sulfur mustard vapor in air, corresponding with 1 LCt50.

	10 min	30 min	60 min	120 min	240 min[a]	24 h[b]	Control
Nasal mucosa	38 ± 18	16 ± 3	17 ± 13	33 ± 18	*56 ± 12*	5 ± 4	0
Nasopharynx	40 ± 15	25 ± 20	37 ± 17	47 ± 13	*69 ± 11*[b]	4 ± 1	0
Larynx	41 ± 12	48 ± 15	49 ± 14	47 ± 12	*88 ± 1*	19 ± 11	0
Trachea	53 ± 10	47 ± 15	34 ± 15	44 ± 13	*90 ± 2*	16 ± 7	0
Carina	41 ± 15	51 ± 16	46 ± 14	41 ± 16[b]	*52 ± 8*[b]	2.7 ± 0.8	0
Lung	3 ± 3	1.6 ± 0.6	1.1 ± 0.5	8 ± 4	*0.3 ± 0.1*	0.05 ± 0.04	0

[a] Data from DAMD17-94-V-4009 (Langenberg *et al.* 1998)
[b] n = 3

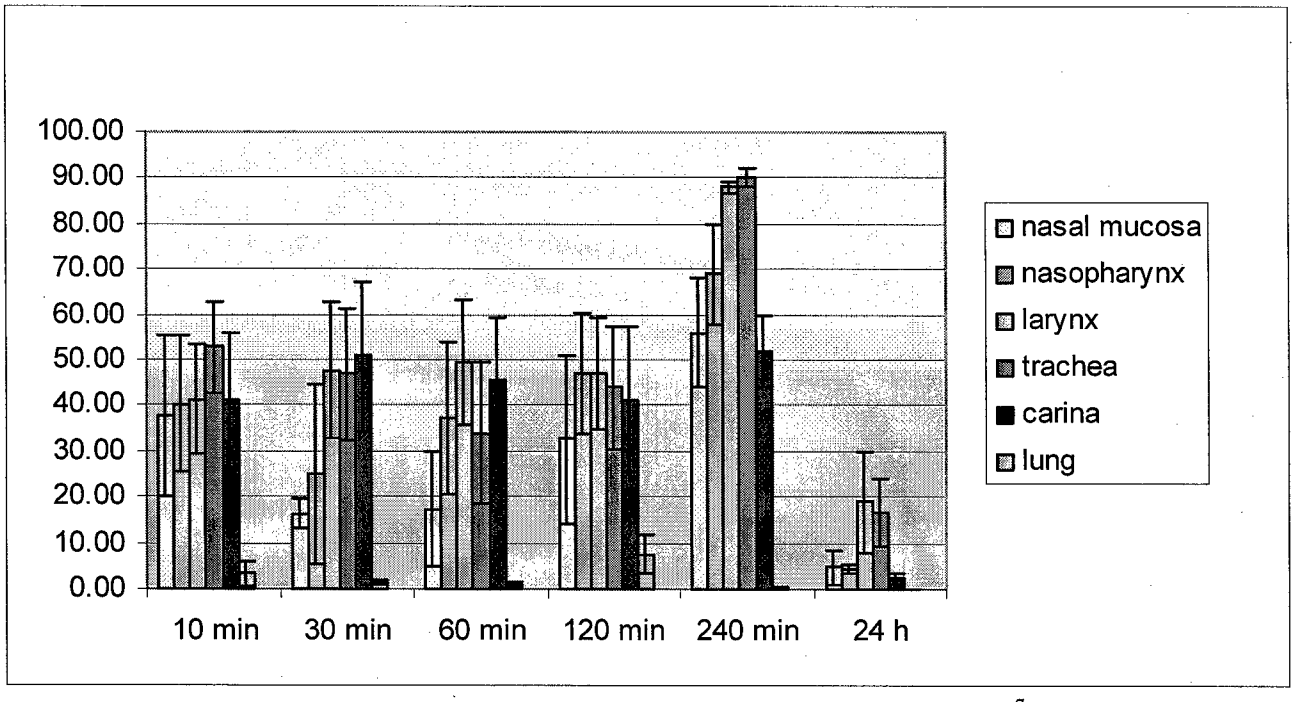

Figure 1. Mean concentration of N7-HETE-Gua (expressed as number of adducts per 10^7 nucleotides) in nasal cavity, nasopharynx, larynx, trachea and carina and lung tissue of hairless guinea pigs at various time points after exposure of sulfur mustard vapor in air (160 mg.m^{-3}, corresponding with 1 LCt50 (96-h)). Error bars represent the SEM (standard error of the mean at $p \leq 0.05$, n=4).

III.2 TIME COURSE OF N7-HETE-GUA IN THE RESPIRATORY TRACT OF HAIRLESS GUINEA PIGS AFTER 5-MIN NOSE-ONLY EXPOSURE TO 0.3 LCT50 SULFUR MUSTARD IN AIR (T.O. 2)

Hairless guinea pigs were weighed, anesthetized and 5-min nose-only exposed to sulfur mustard vapor in air, at a concentration of 48 $mg.m^{-3}$. This results in an exposure dose of 240 $mg.min.m^{-3}$, which corresponds with 0.3 LCt50 (96-h), as determined within the context of Cooperative Agreement DAMD17-94-V-4009 (Langenberg *et al.* 1998). Animals were euthanized at various time points, i.e., at 10 and 30 min, and at 1, 2, 4 and 24 h after ending the 5-min nose-only exposure. For each time point n=4. Samples from the mucosa of various sites of the respiratory tract, i.e., from the nasal cavity, nasopharynx, larynx, trachea and carina, were collected by scraping off the mucosal surfaces. Furthermore, the lungs were sampled. In all of these samples the amount of adducts of sulfur mustard to guanine in the DNA was determined.
Generation and monitoring of sulfur mustard vapor in air are described in paragraph III.1.
Due to the limitations (in terms of resolution) of the 12-bit data-acquisition board, the reported vapor concentration seems to fluctuate quite a bit. In reality the concentration cannot vary that much, as verified by manual re-integration of the chromatographic peaks. The relatively small standard deviation of these calculations of the vapor concentration emphasizes the overall quality of the analytical configuration.
An example of the time course of the sulfur mustard vapor concentration can be seen in Figure 2.
In Table 4 the individual averaged vapor concentrations as well as the deviation of these concentrations are presented.

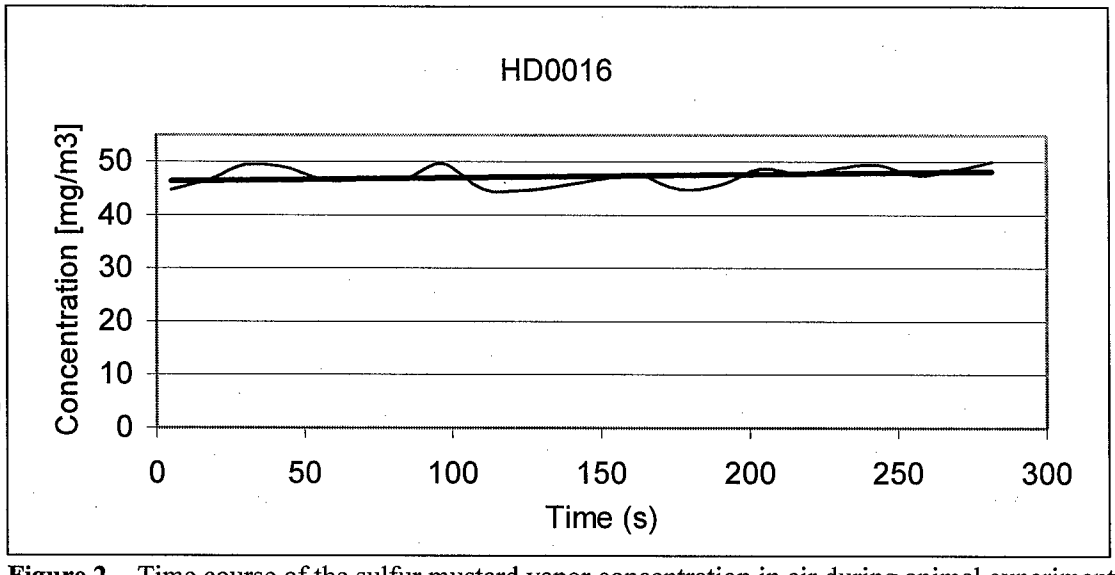

Figure 2. Time course of the sulfur mustard vapor concentration in air during animal experiment HD0016.

Distribution of N7-HETE-Gua within the respiratory tract of the hairless guinea pig after 5-min nose-only exposure to sulfur mustard vapor in air of 0.3 LCt50.
The concentration of N7-HETE Gua was measured in the respiratory tract samples with the immuno-slot-blot procedure described in paragraph II.2 and Annex B. Immunodetection of N7-HETE-Gua has proven to be a sensitive, reproducible and reliable method to quantify damage caused by sulfur mustard (Benschop *et al.* 1997, van der Schans *et al.* 1996, Langenberg *et al.*, 1998).
The concentrations of N7-HETE-gua measured in the respiratory tract samples of the individual animals as well as the averaged values with SEM per group of 4 animals for each time point are presented in Tables 5 and 6, respectively. The distribution of N7-HETE-Gua within the respiratory tract at the various time points after exposure is presented in Figure 3.

Table 4 The concentration of sulfur mustard vapor in air to which the individual animals were exposed.

Guinea pig #	Weight (g)	Concentration ($mg.m^{-3}$)	S.D. ($mg.m^{-3}$)	Sampling time (min)
HD 001	496	50.1	2.9	10
HD 002	523	47.1	2.2	10
HD 003	552	49.0	1.6	10
HD 004	596	49.6	2.4	10
Average	542	49.0		
HD 005	533	49.5	2.0	30
HD 006	527	47.4	2.9	30
HD 007	545	48.9	1.5	30
HD 008	578	48.4	3.1	30
Average	546	48.6		
HD 009	532	48.5	3.1	60
HD 010	527	48.9	1.7	60
HD 011	580	48.0	1.5	60
HD 012	573	47.3	1.8	60
Average	553	48.2		
HD 013	513	50.7	1.6	120
HD 014	504	50.5	2.0	120
HD 015	565	47.9	1.9	120
HD 016	608	48.6	2.0	120
Average	548	49.4		
HD 017	486	47.7	3.4	240
HD 018	502	47.9	1.5	240
HD 019	529	50.0	2.4	240
HD 020	516	49.3	2.5	240
Average	508	48.7		
HD 021	542	50.2	1.9	24
HD 022	532	47.9	1.5	24
HD 023	563	47.6	1.7	24
HD 024	592	48.3	1.7	24
Average	557	48.5		
Average	542	48.7	1.1	

Table 5. N7-HETE-gua (expressed as number per 10^7 nucleotides) in respiratory tract regions of individual hairless guinea pigs at various time points after 5-min nose-only exposure to 48 $mg.m^{-3}$ of sulfur mustard vapor in air, corresponding with 0.3 LCt50.

	10 min	*30 min*	*60 min*	*120 min*	*240 min*	*24 h*	*control*

	HD001	HD005	HD009	HD013	HD017	HD021	HD025
Nasal mucosa	6.65	0.14	7.3	2.31	3.76	17.33	0
Nasopharynx	57.64	42.43	3.92	7.32	2.86	0	0
Larynx	48.06	16.78	23.42	27.44	2.77	2.67	0
Trachea	31.51	33.04	13.99	13.55	1.34	4.2	0
Carina	n.m.	18.77	n.m.	8.27	3.85	2.7	0
Lung	0	0	0	0	0	0	0

	HD002	HD006	HD010	HD014	HD018	HD022	HD026
Nasal mucosa	0	8.54	0	0.03	0	0	0
Nasopharynx	5.75	11.32	8.07	6.5	27.19	3.6	0
Larynx	0.13	1.87	1.55	0	15.73	3.3	0
Trachea	3.32	0	1.41	2.78	0	2.58	0
Carina	2.34	3.08	8.42	5.25	n.m.	4.57	0
Lung	0	0	0	0	0	0	0

	HD003	HD007	HD011	HD015	HD019	HD023	HD027
Nasal mucosa	0.7	0	0	0	0	0	0
Nasopharynx	0	0	n.m.	0	n.m.	n.m.	
Larynx	0	0.16	n.m.	n.m.	n.m.	0	0
Trachea	0.38	11.24	0.42	1.12	9.66	2.63	0
Carina	8.38	8.19	4	0.56	10.01	1.25	
Lung	0	0.01	0	0	0	0	0

(remeasured)	HD003	HD007	HD011	HD015	HD019	HD023	HD027
Nasal mucosa	0	2.74	9.76	5.02	5.22	2.25	0
Lung	0	0	0	0	0	0	0

	HD004	HD008	HD012	HD016	HD020	HD024	HD028
Nasal mucosa	0.08	14.81	0.21	0.28	0	0	0
Nasopharynx	10.38	9.47	16.21	8.53	12.65	2.71	0
Larynx	6.77	13.61	3.26	3.77	3.13	0.65	0
Trachea	7.58	33.21	5.77	4.34	5.69	2.96	0
Carina	9.48	4.6	3.35	2.76	3.19	0	0
Lung	0	0	0	0	0	0	0

n.m. = not measured

Table 6. Mean concentrations of N7-HETE-gua (expressed as number per 10^7 nucleotides) with SEM (n=4 unless stated otherwise) in respiratory tract regions of individual hairless guinea pigs at various time points after 5-min nose-only exposure to 48 $mg.m^{-3}$ of sulfur mustard vapor in air, corresponding with 0.3 LCt50.

	10 min	30 min	60 min	120 min	240 min	24 h	control
Nasal mucosa	1.5 ± 1.3	5.2 ± 2.8	3.4 ± 2.1	1.5 ± 1.0	1.8 ± 1.1	3.9 ± 3.4	0
Nasopharynx	18 ± 13	16 ± 9.2	9.4 ± 3.6[a]	5.6 ± 1.9	14 ± 7[a]	2.1 ± 1.1[a]	0
Larynx	14 ± 12	8.1 ± 4.2	9.4 ± 7.0[a]	10 ± 9[a]	7.2 ± 4.3[a]	1.7 ± 0.8	0
Trachea	11 ± 7.1	19 ± 8.3	5.4 ± 3.1	5.4 ± 2.8	4.2 ± 2.2	3.1 ± 0.4	0
Carina	6.7 ± 2.2[a]	8.7 ± 1.5	5.3 ± 1.6[a]	4.2 ± 1.4	5.7 ± 2.2[a]	2.1 ± 1.4	0
Lung	0	0	0	0	0	0	0

[a] n = 3

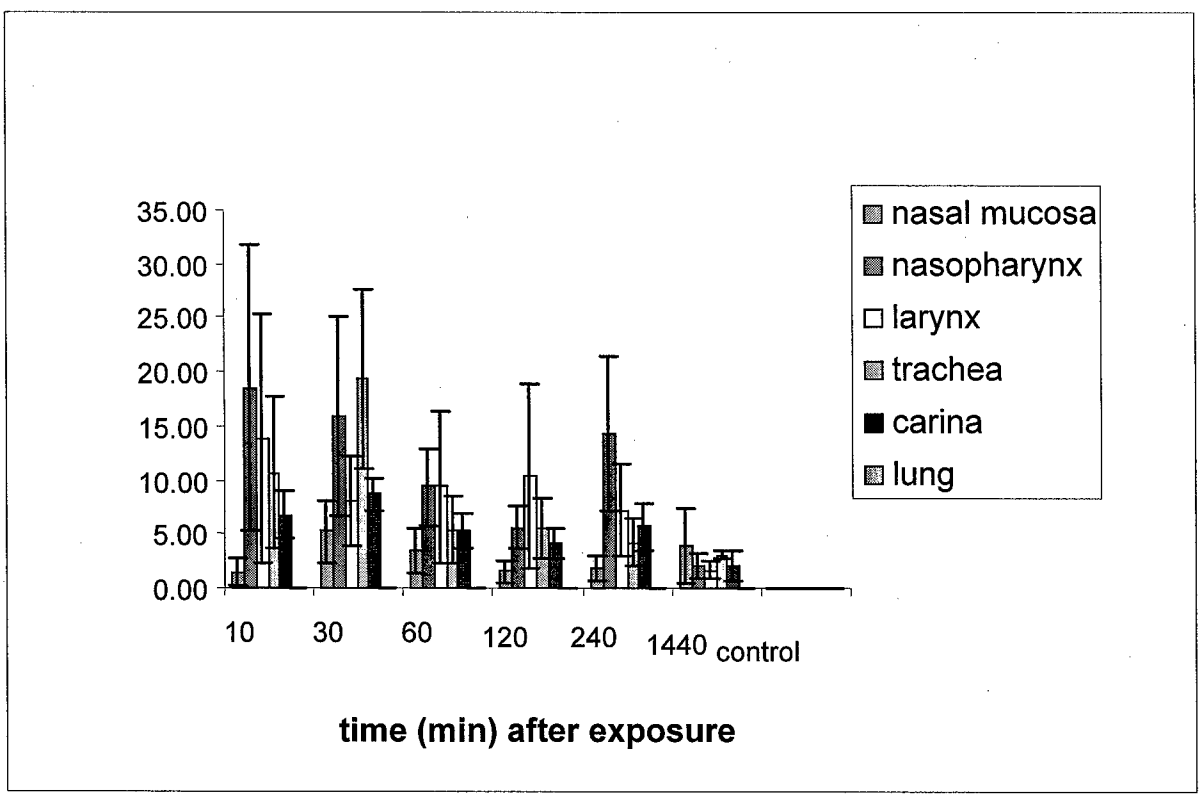

Figure 3. Mean concentration of N7-HETE-Gua (expressed as number of adducts per 10^7 nucleotides) in nasal cavity, nasopharynx, larynx, trachea and carina and lung tissue of hairless guinea pigs at various time points after exposre of sulfur mustard vapor in air (48 $mg.m^{-3}$, corresponding to 0.3 LCt50 (96-h)). Error bars represent the SEM (standard error of the mean at $p \leq 0.05$, n=4).

N7-HETE-Gua could be detected in the nasal cavity, nasopharynx, larynx, trachea and carina, but not in lung tissue following 5-min nose-only exposure to 0.3 LCt50 of sulfur mustard vapor in air. The highest adduct levels were found at 10 and 30 min after exposure in the nasopharynx (up to 18 ± 13 (SEM, $p \leq 0.05$, n=4) adducts per 10^7 nucleotides) and trachea (up to 19 ± 8 adducts per 10^7 nucleotides). The lowest adduct levels were found in the nasal mucosa at all time points after exposure (2-5 ± SEM adducts per 10^7 nucleotides), and in all respiratory organs, except for lung tissue, at 24 h after exposure.

In the nasopharynx the highest adduct level is measured at 10 min after ending the exposure, after which it decreases with time, most likely due to repair of the damage to DNA. Deeper in the respiratory tract, i.e., in the trachea the maximum adduct level is observed at 30 min after the level decreases.
In general the adduct levels appear to build up relatively rapidly after nose-only exposure to sulfur mustard vapor in air and decrease quite rapidly within the first 24 h after ending the exposure. This observation is in agreement with previous findings (Langenberg *et al.* 1998).

III.3 APPARATUS FOR NOSE-ONLY EXPOSURE OF MARMOSETS TO SULFUR MUSTARD VAPOR IN AIR (T.O. 3)

In view of the anatomy of the marmoset, the modified Battelle tube for nose-only exposure of hairless guinea pigs as developed within the context of Cooperative Agreement DAMD17-94-V-4009 (Langenberg *et al.* 1998) is not suitable for this species. Consequently, a specially designed stainless steel 'chair' was developed. In this chair the animal can be restrained in such a way that it can breathe freely and cannot free its arms or legs. The chair is adjustable for smaller and larger animals. The armrest, the chair support and the leg support are designed in such a way that they can be adapted easily to the individual animal. Special attention has been paid to the head support. Due to the physiology of the animal, to breathe freely the angle between the mouth-head and head-rest of the body is slightly less than 90°, but can be adapted as desired. Figures 4-6 are photographs of a marmoset in the chair. Figures 7 and 8 show how the chair is connected to the exposure module.

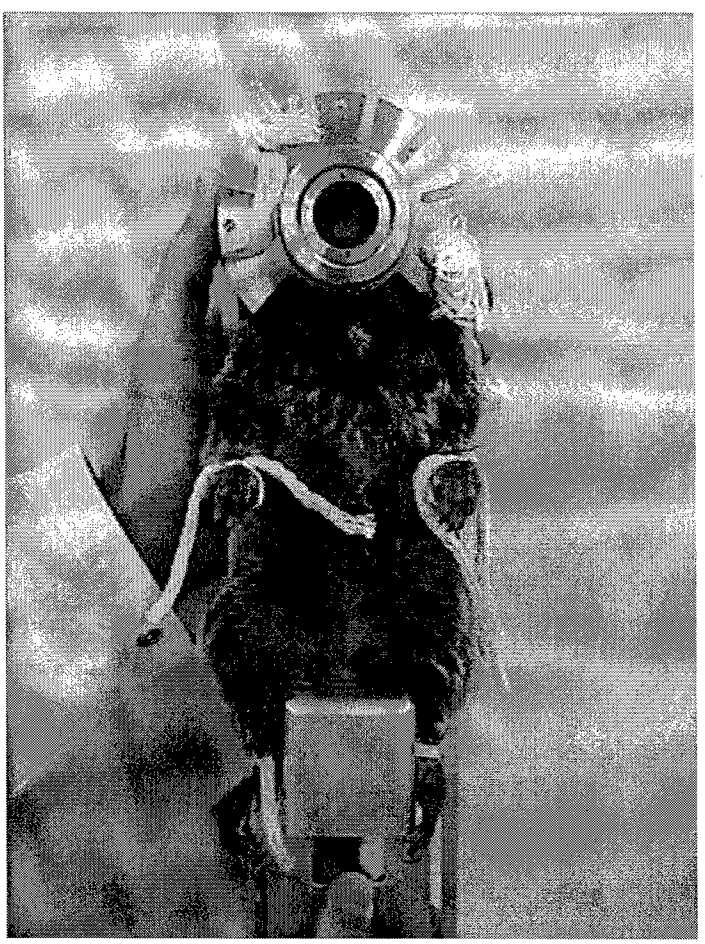

Figure 4. Photograph of a marmoset monkey restrained on the chair, front view.

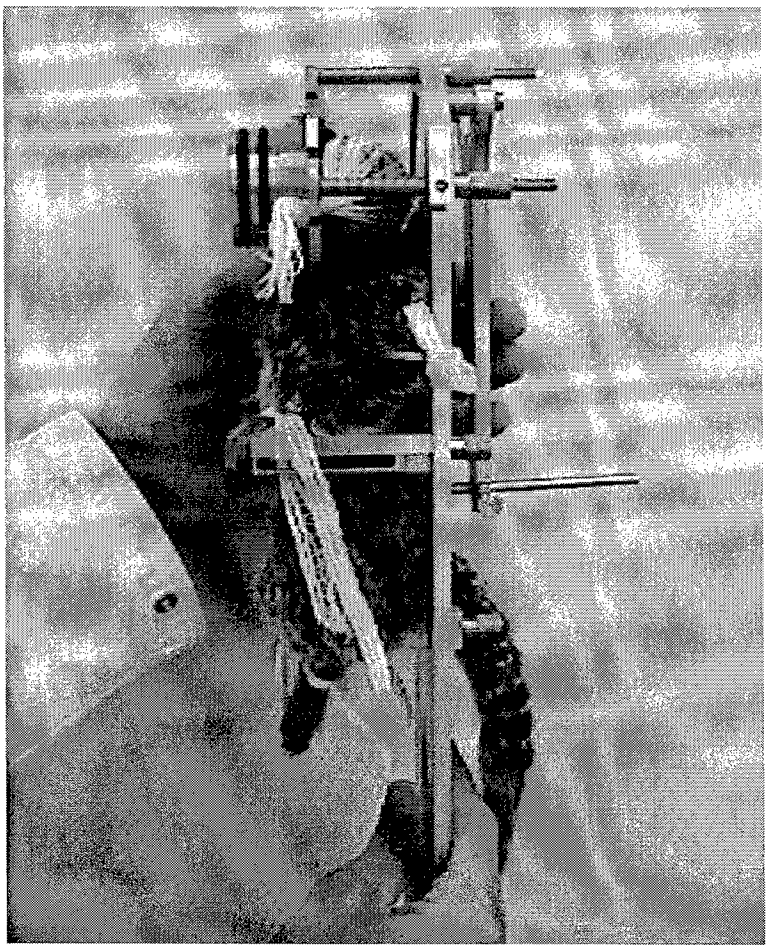

Figure 5. Photograph of a marmoset monkey restrained on the chair, side view.

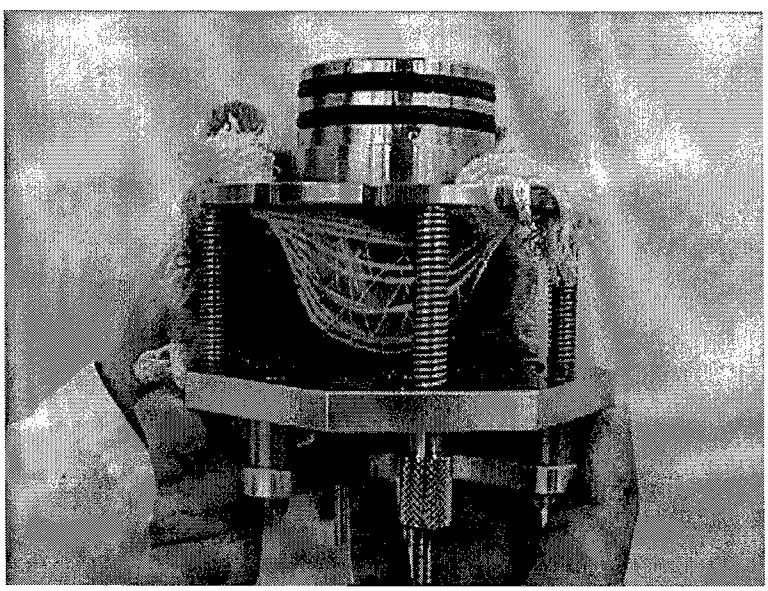

Figure 6. Photograph of a restrained marmoset on the chair, top view. The angle of the mouthpiece (top) to the body is adjustable.

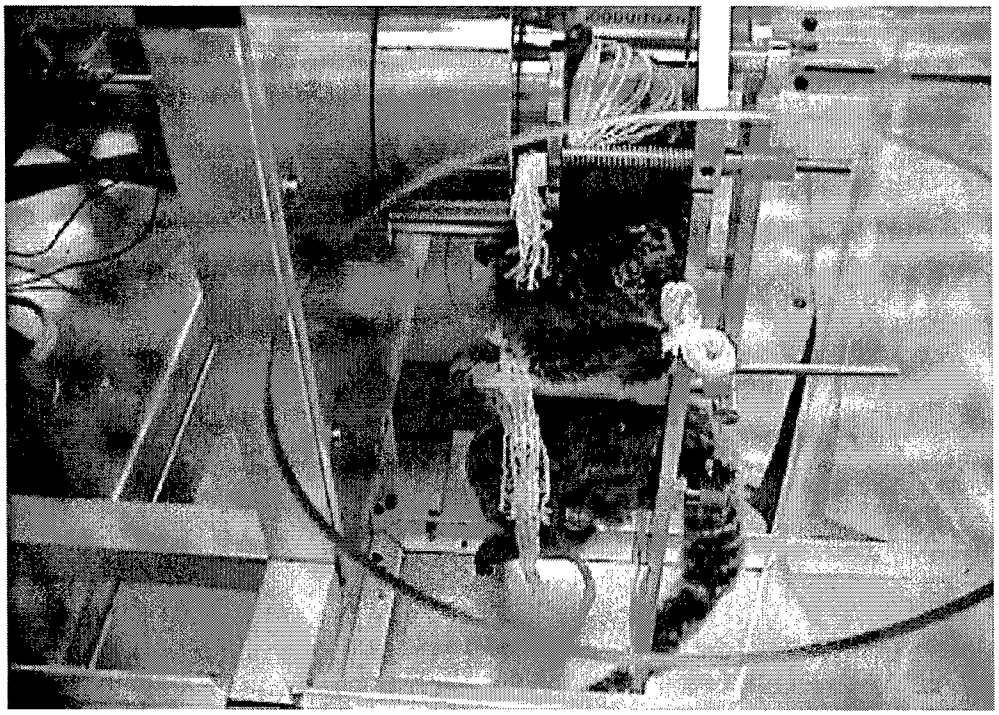

Figure 7. Photograph of a marmoset monkey fixated in the 'chair' and connected into the 'mouth-piece' of the exposure module, taken from the left hand side.

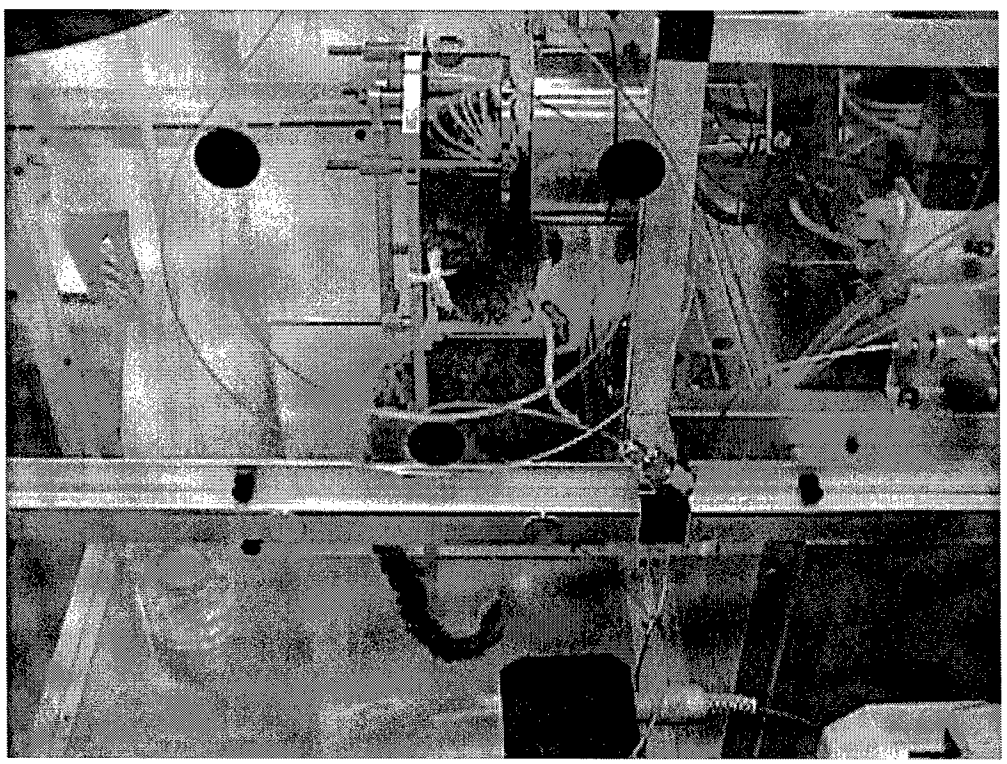

Figure 8. Photograph of a marmoset monkey fixated in the 'chair' and connected into the 'mouth-piece' of the exposure module, taken from the right hand side.

III.4 INHALATION TOXICOKINETICS OF SULFUR MUSTARD IN THE ANESTHETIZED MARMOSET (T.O. 4)

III.4.1. Improvement of the analytical procedure for sulfur mustard in blood (T.O. 4.1)

Within the context of Cooperative Agreement DAMD17-94-V-4009 a method was developed for analysis of sulfur mustard in blood and tissues of the hairless guinea pig, using gas chromatography with mass-selective detection (EI) and large volume injection (thermodesorption from tenax TA) (Langenberg *et al.* 1998). The method proved to be adequate for that purpose, but a drawback was the high background at *m/z* 109, which had an adverse effect on the detection limit. Since extremely low concentrations of sulfur mustard were anticipated in the blood of marmosets during and after nose-only exposure to sulfur mustard vapor in air, and the number of marmosets to be used had to be as small as reasonably possible, it was decided to try and and improve the selectivity and sensitivity of the analytical procedure. Upon revision of the Statement of Work a Technical Objective (T.O. 4.1) addressing this aspect was included.

Firstly, an Agilent micro electron capture detector (μECD) was tested. The detection limit for D8-sulfur mustard was considered to be insufficient. Next, an Agilent flame-photometric detector (FPD) with sulfur filter was tested, but also this detector was not sensitive enough (780 pg D8-sulfur mustard with an S/N ratio of 21).
Apparently the ECD and FPD technologies have not evolved much over the past 10 years, since similar conclusions were drawn in the previous study (Langenberg *et al.* 1998).

Next, various MSD detection modes were studied, using the Agilent MSD 5973 as a detector. As a reference (EI), a 1-μl D8-sulfur mustard injection of 26 pg (SIM) resulted in an S/N-ratio of 23 (*m/z* 115) and in the full scan mode 26 ng of this compound resulted in an S/N-ratio (TIC) of 1,063. Although this is an adequate absolute sensitivity for the pure substance, there was a lack of selectivity as mentioned above.
In the negative and positive chemical ionisation modes (NCI/PCI), the following results were obtained:

NCI-CH_4:	Full scan, 7.8 ng D8-sulfur mustard, no molecular ion, strong fragmentation, most abundant ion: *m/z* 35 (Cl^-), also present in the methane background, S/N 2.
NCI-NH_3:	Full scan, 7.8 ng D8-sulfur mustard, no molecular ion, *m/z* 35 (Cl^-) present, also present in ammonia background, S/N (TIC) 4.
PCI-CH_4:	Full scan, 78 ng D8-sulfur mustard, low fragmentation, molecular ion *m/z* 167 (MH^+) present, most abundant ion (50% of TIC): *m/z* 131 (MH^+-HCl), S/N (TIC) 160.
PCI-C_4H_{10}:	Full scan, 78 ng D8-sulfur mustard, low fragmentation, molecular ion *m/z* 167 (MH^+) present, most abundant ion (40% of TIC): *m/z* 167, S/N (TIC) 160.
PCI-NH_3:	Full scan, 78 ng D8-sulfur mustard, strong fragmentation, no molecular ion present, most abundant ion: *m/z* 131 ((MH^+-HCl), S/N (TIC) 33.
PCI-Ar:	Full scan, 7.8 ng D8-sulfur mustard, strong fragmentation, molecular ion *m/z* 167 (MH^+) present, most abundant ion: *m/z* 67 (M-S-CD_2-CD_2-Cl)$^+$, S/N (TIC) 14.

It was concluded that none of the MSD detection modes studied provides the required combination of selectivity and sensitivity. Consequently, it was decided to continue with MSD in the EI mode, which is sufficiently sensitive, and try and obtain more selectivity via modification of the gas chromatographic procedure. For this purpose the Multiple Column Switching Intelligent Conroller (MuSIC) column switching system (Chrompack/Varian) was implemented into the system. The MuSIC system is described earlier by Langenberg *et al.* (1998) and has been used successfully in the analysis of ultra-trace levels of nerve agent stereoisomers in the past (Benschop and De Jong 1988; Benschop *et al.* 1993).
Next, the conditions for two-dimensional gas chromatography were optimized. An FPD was used to establish the optimal conditions for chromatography on the pre-column and the fraction to be trapped. Conditions for the analytical column were optimized using the MSD. The conditions to be used are listed in paragraph II.3 (*Configuration 2*).

Some photographs of the TDAS-MuSIC-GC-MSD are shown in Figure 9.

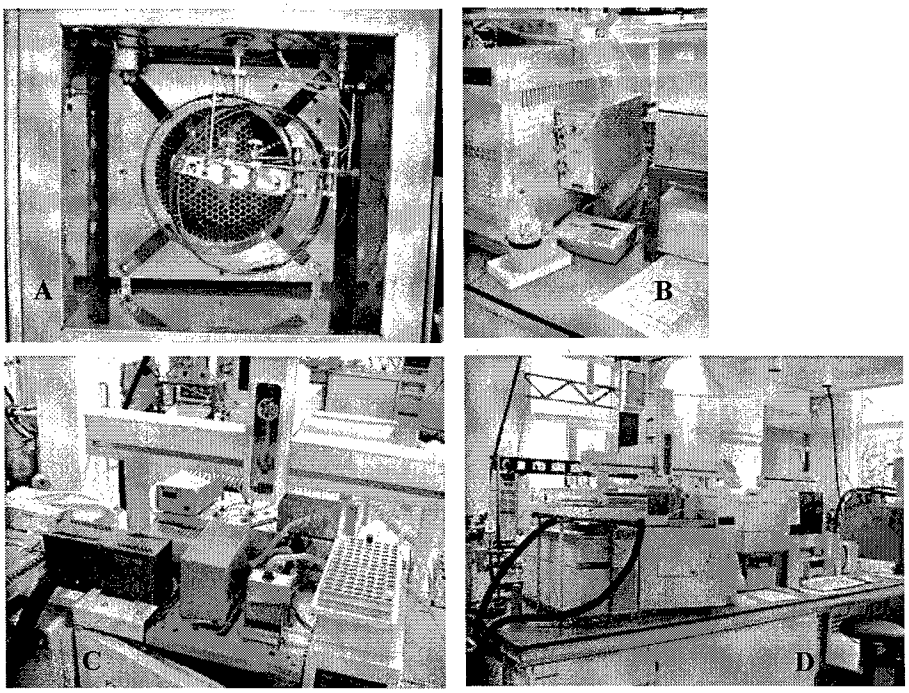

Figure 9 Photographs of gas chromatographic configuration 2 (TDAS-MuSIC-GC-MSD) A. Oven with pre-column and analytical column; B. MuSIC unit; C. TDAS, MuSIC cold trap and FPD; D. Overview of the complete system.

To show the positive effect of the column-switching system on the detection limit of sulfur mustard a 500-µl blood sample of marmoset M001 (drawn at t=20 min) was worked-up and analyzed both on configuration 1 (TDAS-GC-MSD) and configuration 2 (TDAS-MuSIC-GC-MSD). From the ethyl acetate extract from blood, 400 µl was transferred onto Tenax, containing *ca.* 800 pg D8-sulfur mustard and 24 pg sulfur mustard. Figure 10 shows that the S/N ratio is less important than the 'chemical noise' from the background: with configuration 1 the sulfur mustard peak drowns in the background signal and therefore cannot be analyzed. With configuration 2, however, there is enough selectivity and sensitivity to analyse sulfur mustard at 24 pg level in a complex matrix.

Although the system is amenable to contamination a sensitive and selective analysis is possible in this way down to 10-20 pg sulfur mustard per ml of blood. For tissue samples (and large volumes of blood), the detection limit could be somewhat higher due to background interference from the sample. Mainly fat samples caused problems due to rapid contamination of the system, requiring frequent (preventive) replacement of the transfer line, liner and the first part (30 cm) of the pre-column.
The ratio of *m/z* 109/111 [target/qualifier (T/Q)] was used as a positive indicator for the presence of the sulfur mustard peak. If the T/Q ratio was correct, the peak height ratio of *m/z* 109 and 115 was used for quantitation of sulfur mustard in the sample.

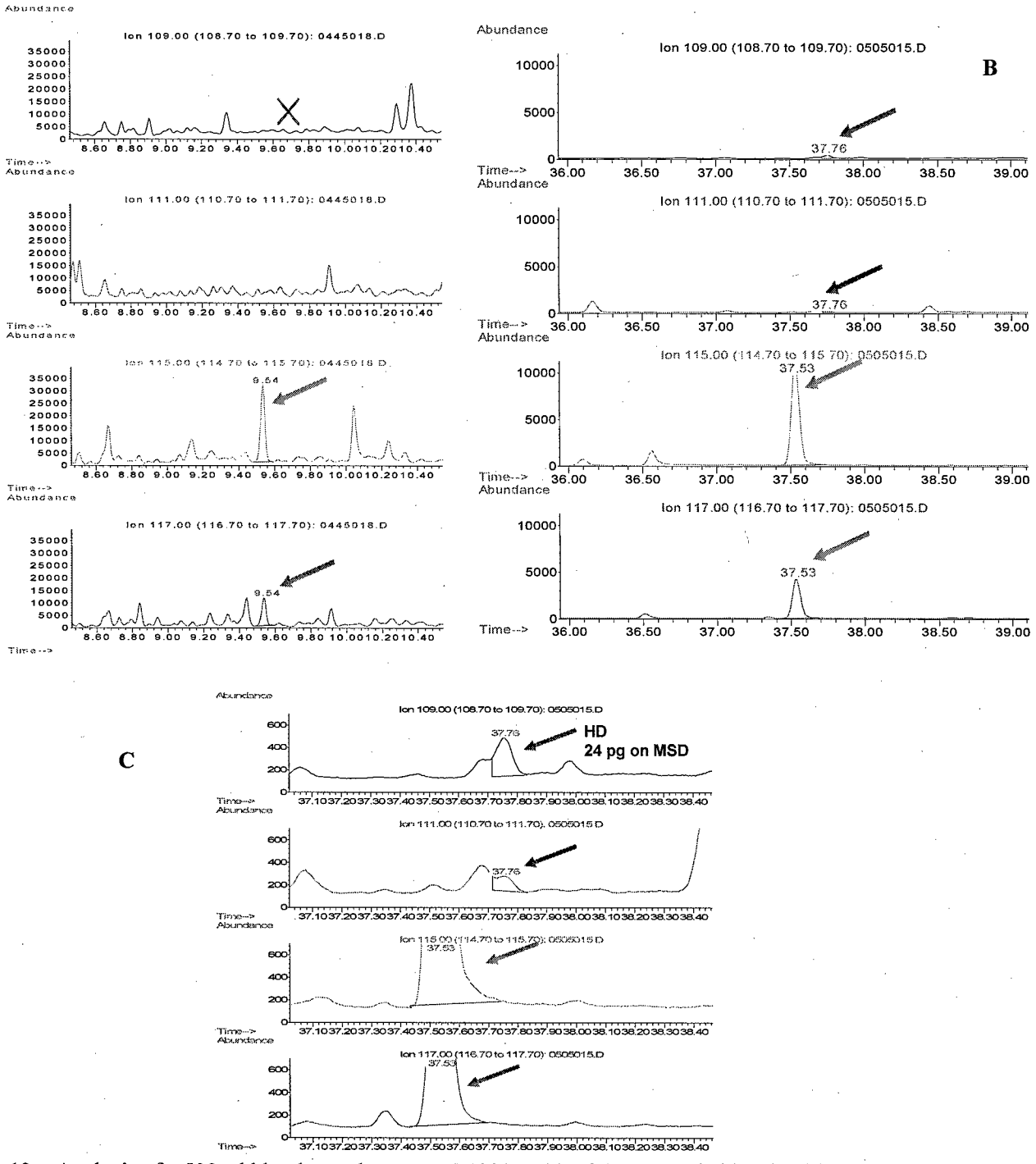

Figure 10. Analysis of a 500-µl blood sample extract (M001, t=20 min) at 69 pg/ml level, with and without a column-switching system (sulfur mustard at *m/z* 109 and 111, D8-sulfur mustard at *m/z* 115 and 117). A. Configuration 1, 800 pg D8-sulfur mustard (tr 9.54 min) (green arrow), no sulfur mustard detected (ca 9.7 min); B. Configuration 2, column-switching system, D8-sulfur mustard at tr 37.53 min and sulfur mustard at tr 37.76 min (red arrow); C. details of the sulfur mustard (HD) peak, just resolved from an unknown impurity, with configuration 2. See text for further details.

III.4.2. Blood toxicokinetics of sulfur mustard in the anesthetized marmoset during and after 5-min nose-only exposure to 160 mg.m^{-3}, which corresponds with 1 LCt50 (96-h) in the hairless guinea pig (T.O. 4)

Anesthetized, restrained marmosets were nose-only exposed to sulfur mustard in air at a concentration of *ca.* 160 mg.m^{-3} for 5 min, yielding a Ct-value of 800 mg.min.m^{-3}, which was previously determined to correspond with 1 LCt50 (96-h) in the hairless guinea pig (Langenberg *et al.* 1998). The actual average concentrations of sulfur mustard in air during the 5-min exposure period for the individual animals are presented in Table 7.

Table 7. The average concentration of sulfur mustard vapor in air to which the individual animals were 5-min nose-only exposed.

Marmoset #	Gender	Weigth (g)	Mean concentration ± S.D. (mg.m^{-3})	Experiment terminated at (min)
M001	M	360	162 ± 7	120
M002	M	395	163 ± 10	60
M003	F	350	166 ± 19	60
M004	M	297	160 ± 9	60
M005	F	320	162 ± 10	60
M006	F	318	160 ± 10	60
M007	M	304	164 ± 7	60

Sulfur mustard was measurable in the blood samples taken from anesthetized and restrained marmosets during and after the 5-min exposure period.

The results obtained for animal M001 are presented in Table 8. Analysis was performed with GLC configuration 1 (TDAS-GC-MSD), which was clearly not sufficiently selective for this purpose. The detection limit for sulfur mustard when analyzed in standard solutions was sufficiently low (*ca.* 10 pg.ml^{-1}), but due to the background signal of the matrix the actual detection limit in extracts from blood was one order of magnitude higher. The results obtained for animal M001 were rejected and the analytical procedure was improved as described in paragraph III.4.1.

Table 8. Concentrations of intact sulfur mustard (pg.ml^{-1}) measured in blood samples drawn from marmoset M001 during and after 5-min nose-only exposure to 162.2 mg.m^{-3} of sulfur mustard vapor in air.

Time (min)	Sulfur mustard concentration (pg.ml^{-1})	Blood sample volume (µl)
0	n.d.	300
1	13,374	300
5	n.d.	500
10	2,838	500
15	342	500
20	n.d.	500
30	n.d.	500
60	n.d.	500
90	n.d.	500
120	n.d.	500
>120	n.d.	4,000

n.d.: not detectable (< *ca.* 100 pg.ml^{-1})

The concentrations of intact sulfur mustard measured in animals M002-M007 are presented in Table 9

Table 9. Concentrations of intact sulfur mustard ($pg.ml^{-1}$) measured in blood of individual marmosets during and after 5-min nose-only exposure to 160 $mg.m^{-3}$ of sulfur mustard vapor in air. Mean concentrations ± SEM ($ng.ml^{-1}$) are also presented.

	M 002	M 003	M 004	M 005	M 006	M 007		
Sample time (min)	$pg.ml^{-1}$	$pg.ml^{-1}$	$pg.ml^{-1}$	$pg.ml^{-1}$	$pg.ml^{-1}$	$pg.ml^{-1}$	Mean concentration ± SEM ($ng.ml^{-1}$)	Sample volume (µl)
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	100
1	2,960	153	2,097	1,893	1,909	118	1.52 ± 0.47	200
3	30,040	1,541	28,685	9,872	10,595	416	13.5 ± 5.3	100
5	58,276	6,017	62,080	20,517	25,662	1,429	29.0 ± 10.5	100
7.5	23,314	930	21,989	4,636	81,88	1,207	10.0 ± 4.1	300
10	11,714	165	7,047	574	2,253	329	3.68 ± 1.90	500
15	1,786	57	426	162	282	55	0.461 ± 0.271	500
20	71	19	213	70	111	30	0.086 ± 0.029	750
30	206	13	109	39	62	12	0.086 ± 0.030	1,000
45	219	11	115	28	38	10	0.070 ± 0.034	1,000
60	210	17	55	23	28	10	0.057 ± 0.031	*ca.* 6,000

n.d. = below detection limit (< ca 10 $pg.ml^{-1}$)
Measured with GLC configuration 2

Interestingly, a complete curve could be measured in each animal. Therefore only 6 animals were needed to construct a mean concentration-time curve with n=6. This curve is shown in Figure 11. The concentration-time curves for the individual animals are presented in Figures 20-27 in ANNEX C.

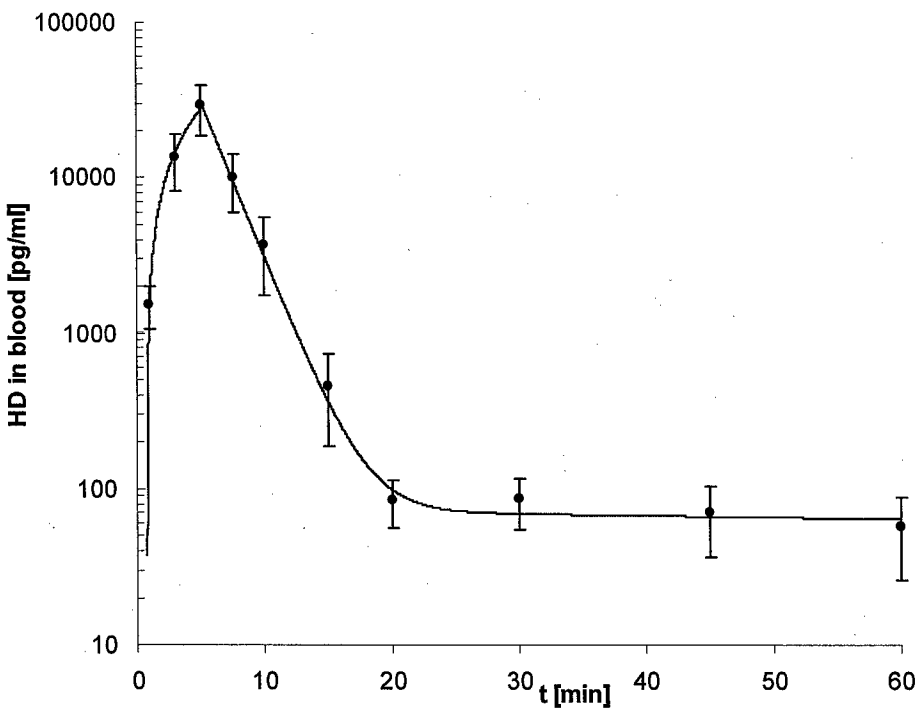

Figure 11. Mean concentration-time curve with SEM (n=6) of sulfur mustard (HD, $pg.ml^{-1}$) in the blood of anesthetized, restrained marmosets during and after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

The absorption phase (0-5 min) was described with a mono-exponential equation, and the post-exposure curve (5-60 min) with a bi-exponential equation. The toxicokinetic parameters derived from the curve are presented in Table 10.

Table 10. Toxicokinetic parameters[a] of sulfur mustard in anesthetized and restrained marmosets 5 min nose-only exposed to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

Parameter	Dimension	Value
Ct	$mg.min.m^{-3}$	800
A	$ng.ml^{-1}$	-4.92
B	$ng.ml^{-1}$	6.44
C	$ng.ml^{-1}$	320
D	$ng.ml^{-1}$	0.074
c	min^{-1}	0.467
d	min^{-1}	0.00246
$AUC_{0\rightarrow\infty}$	$ng.min.ml^{-1}$	740
$t_{½,c}$	min	1.5
$t_{½,d}$	min	280

[a]The results were fitted with a discontinuous function: [sulfur mustard] = A +B*t for the absorption phase (0-5 min) and [sulfur mustard] = $C*e^{-ct} + D*e^{-dt}$ for the distribution and elimination phase (>5 min).

III.4.3. Toxicokinetics of sulfur mustard in various tissues of the anesthetized marmoset at various time points after 5-min nose-only exposure to 160 $mg.m^{-3}$, which corresponds with 1 LCt50 (96-h) in the hairless guinea pig (T.O. 5)

Marmosets were nose-only exposed to 160 $mg.m^{-3}$ sulfur mustard vapor in air for 5 min and then euthanized at various time points. The average vapor concentration to which the animals were exposed and the end time of the experiment are listed for the individual animals in Table 11.

Table 11. The average concentration of sulfur mustard vapor in air to which the individual animals were 5-min nose-only exposed.

Marmoset number	Gender	Weight g)	Concentration ($mg.m^{-3}$)	S.D. ($mg.m^{-3}$)	End time (min)
M 008	F	390	163.7	9	10
M 009	M	473	166.4	7	10
M 010	F	450	163.5	8	10
M 011	M	392	163.8	10	24 h
M 012	F	433	161.0	17	24 h
M 013	M	370	162.6	9	24 h
M 014	M	400	158.1	10	10
M 015	F	490	159.2	8	30
M 016	M	374	154.5	19	30
M 017	F	407	160.4	11	24 h
M 018	M	340	158.7	8	30
M 019	F	367	156.0	7	30
M 020	M	379	148.1	8	30
M 021	M	340	159.7	11	30
M 022	M	353	164.3	9	24 h
Average		**380**	**160.8**	**4.2**	

Grey shaded rows: results rejected (see text).

The results of the marmosets M 015, M 017 and M 019 were rejected. These animals, obtained from a German company, died during or shortly after the exposure. Upon autopsy, these females appeared to have large uterine tumors.

The concentrations of intact sulfur mustard measured in the various tissue samples at time points 10 min, 30 min, 60 min and 24 h are presented in Tables 12-15. The mean concentrations with S.D. are summarized in Table 16. Note: the 60-min samples were obtained from the experiments in which the blood toxicokinetics were measured.

Table 12. Concentrations of intact sulfur mustard in tissue samples of the marmoset which were 5-min nose-only exposed to 160 $mg.m^{-3}$ of sulfur mustard vapor in air, taken 10 min after the start of the exposure.

Tissue	Concentration ($pg.g^{-1}$)	Animal number	Vapor concentration ($mg.m^{-3}$)
Lung	7	M 008	163.7
	21	M 009	166.4
	n.d.	M 010	163.5
	n.d.	M 014	158.1
Lung average	**7 ± 10** (S.D. n=4)		
Liver	313	M 008	163.7
	494	M 009	166.4
	506	M 010	163.5
	2,155	M 014	158.1
Liver average	**867 ± 863** (S.D. n=4)		
Spleen	n.d.	M 008	163.7
	10	M 009	166.4
	n.d.	M 010	163.5
	n.d.	M 014	158.1
Spleen average	**3 ± 5** (S.D. n=4)		
Fat	318	M 008	163.7
	264	M 009	166.4
	48	M 010	163.5
	877	M 014	158.1
Fat average	**377 ± 355** (S.D. n=4)		
Bone marrow	1,832	M 008	163.7
	1,017	M 009	166.4
	534	M 010	163.5
	1,462	M 014	158.1
Bone marrow average	**1,211 ± 561** (S.D. n=4)		

n.d = not detectable (< *ca.* 10 $pg.g^{-1}$)

Average concentrations were calculated by considering 'n.d.' to be zero

Table 13. Concentrations of intact sulfur mustard in tissue samples of the marmoset which were 5-min nose-only exposed to 160 $mg.m^{-3}$ of sulfur mustard vapor in air, taken 30 min after the start of the exposure.

Tissue	Concentration ($pg.g^{-1}$)	Animal number	Vapor concentration ($mg.m^{-3}$)
Lung	n.d.	M 016	154.5
	n.d.	M 018	158.7
	n.d.	M 020	148.1
	n.d.	M 021	159.7
Lung average	**n.d.**		
Liver	840	M 016	154.5
	1,930	M 018	158.7
	87	M 020	148.1
	334	M 021	159.7
Liver average	**798 ± 815** (S.D. n=4)		
Spleen	n.d.	M 016	154.5
	n.d.	M 018	158.7
	n.d.	M 020	148.1
	n.d.	M 021	159.7
Spleen average	**n.d.**		
Fat	n.d.	M 016	154.5
	290	M 018	158.7
	n.d.	M 020	148.1
	n.d.	M 021	159.7
Fat average	**72 ± 145** (S.D. n=4)		
Bone marrow	1,999	M 016	154.5
	3,122	M 018	158.7
	113	M 020	148.1
	199	M 021	159.7
Bone marrow average	**1,358 ± 1462** (S.D. n=4)		
Skeletal muscle	n.d.	M 016	154.5
	n.d.	M 018	158.7
	n.d.	M 020	148.1
	n.d.	M 021	159.7
Skeletal muscle	**n.d.**		

n.d = not detectable (< *ca.* 10 $pg.g^{-1}$)

Average concentrations were calculated by considering 'n.d.' to be zero

Table 14. Concentrations of intact sulfur mustard in tissue samples of the marmoset which were 5-min nose-only exposed to 160 $mg.m^{-3}$ of sulfur mustard vapor in air, taken 60 min after the start of the exposure.

Tissue	Concentration ($pg.g^{-1}$)	Animal number	Vapor concentration ($mg.m^{-3}$)
Lung	n.d.	M 002	163.1
	n.d.	M 003	166.4
	n.d.	M 004	159.6
	n.d.	M 007	164.0
Lung average	**n.d.**		
Liver	516	M 002	163.1
	77	M 003	166.4
	1,575	M 004	159.6
	605	M 007	164.0
Liver average	**693 ± 631** (S.D. n=4)		
Spleen	7	M 002	163.1
	n.d.	M 003	166.4
	n.d.	M 004	159.6
	n.d.	M 007	164.0
Spleen average	**2 ± 4** (S.D. n=4)		
Fat	5,396	M 002	163.1
	n.d.	M 003	166.4
	n.d.	M 004	159.6
	253	M 007	164.0
Fat average	**1,412 ± 2,658** (S.D. n=4)		
Bone marrow	12,886	M 002	163.1
	311	M 003	166.4
	4,284	M 004	159.6
	499	M 007	164.0
Bone marrow average	**4,495 ± 5,886** (S.D. n=4)		

n.d. = not detectable (< *ca.* 10 $pg.g^{-1}$)

Average concentrations were calculated by considering 'n.d.' to be zero

Table 15. Concentrations of intact sulfur mustard in tissue samples of the marmoset which were 5-min nose-only exposed to 160 $mg.m^{-3}$ of sulfur mustard vapor in air, taken 24 h after the start of the exposure.

Tissue	Concentration ($pg.g^{-1}$)	Animal number	Vapor concentration ($mg.m^{-3}$)
Lung	n.m.	M 011	163.8
	n.m.	M 012	161.0
	n.m.	M 013	162.6
	n.m.	M 022	164.3
Lung average	**n.m.**		
Liver	n.d.	M 011	163.8
	n.d.	M 012	161.0
	n.m.	M 013	162.6
	n.m.	M 022	164.3
Liver average	**n.d.** (n=2)		
Spleen	n.m.	M 011	163.8
	n.m.	M 012	161.0
	n.m.	M 013	162.6
	n.m.	M 022	164.3
Spleen average	**n.m.**		
Fat	n.m.	M 011	163.8
	n.m.	M 012	161.0
	n.m.	M 013	162.6
	n.m.	M 022	164.3
Fat average	**n.m.**		
Bone marrow	n.d.	M 011	163.8
	n.d.	M 012	161.0
	n.m.	M 013	162.6
	n.m.	M 022	164.3
Bone marrow average	**n.d.** (n=2)		

n.d. = not detectable (< *ca.* 10 $pg.g^{-1}$)
n.m. = not measured
Average concentrations were calculated by considering 'n.d.' to be zero

Table 16. Mean concentrations ($pg.g^{-1}$, ± S.D., n=4 unless stated otherwise) of sulfur mustard in tissues of marmosets at various time points after a 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

Time (min)	Lung	Liver	Spleen	Fat	Bone marrow
10	7 ± 10	867 ± 863	3 ± 5	377 ± 354	1,211 ± 561
30	n.d.	798 ± 817	0	72 ± 145	1,358 ± 1,462
60	n.d.	693 ± 631	2 ± 4	1,413 ± 2,658	4,495 ± 5,886
1440 (24 h)	n.m.	n.d.[a]	n.m.	n.m.	n.d.[a]

n.d. = not detectable (< *ca.* 10 $pg.g^{-1}$)
n.m. = not measured
[a] n=2

The concentration-time courses of intact sulfur mustard in the various tissues are shown in Figures 12-16.

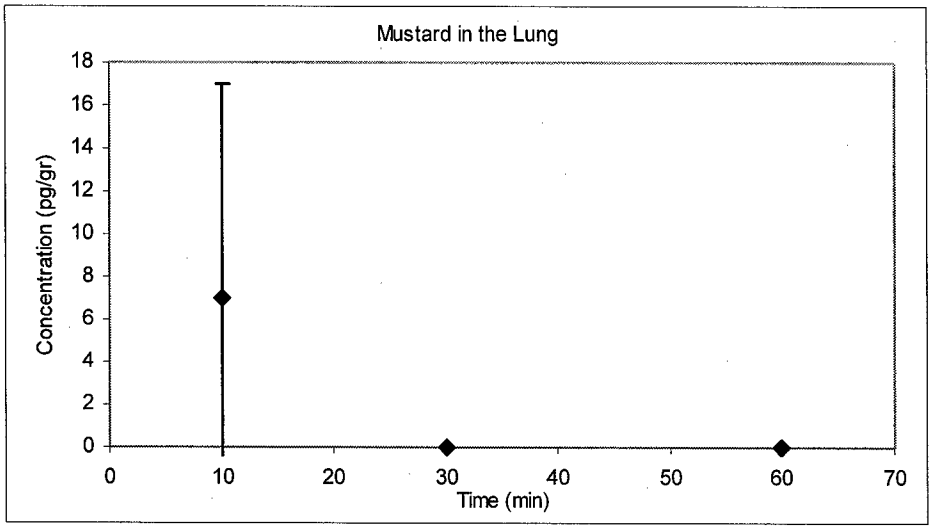

Figure 12. Concentration-time course of intact sulfur mustard (pg.g^{-1}, ± S.D.) in lung tissue of anesthetized, restrained marmosets at various time points after 5-min nose-only exposure to 160 mg.m^{-3} sulfur mustard vapor in air.

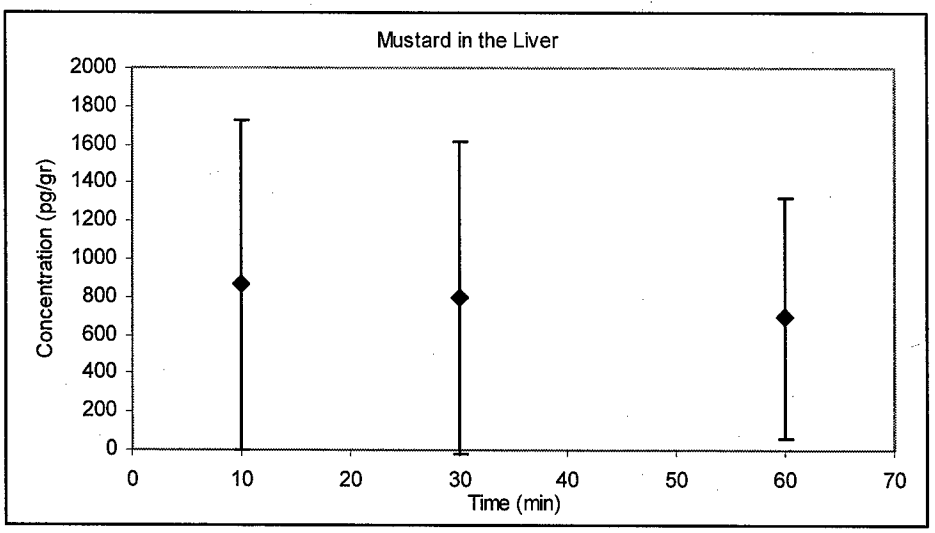

Figure 13. Concentration-time course of intact sulfur mustard (pg.g^{-1}, ± S.D.) in liver of anesthetized, restrained marmosets at various time points after 5-min nose-only exposure to 160 mg.m^{-3} sulfur mustard vapor in air.

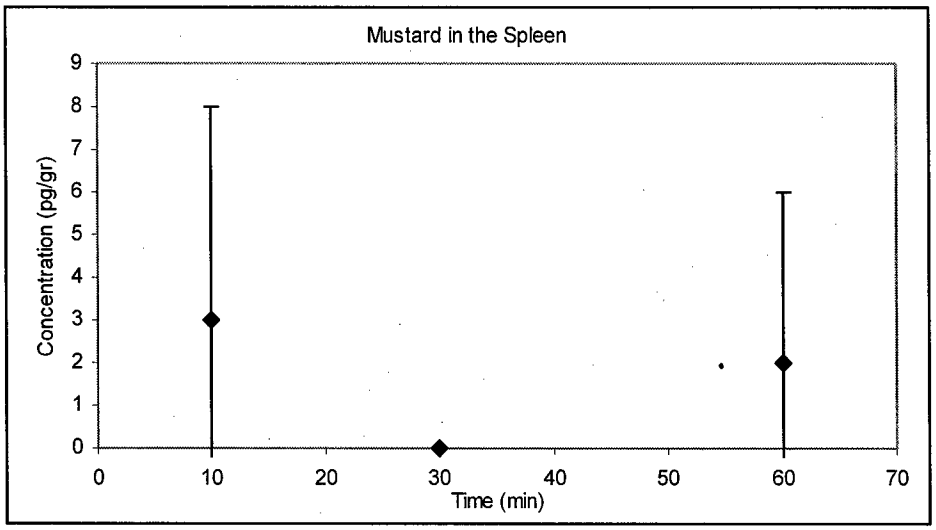

Figure 14. Concentration-time course of intact sulfur mustard ($pg.g^{-1}$, ± S.D.) in spleen of anesthetized, restrained marmosets at various time points after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

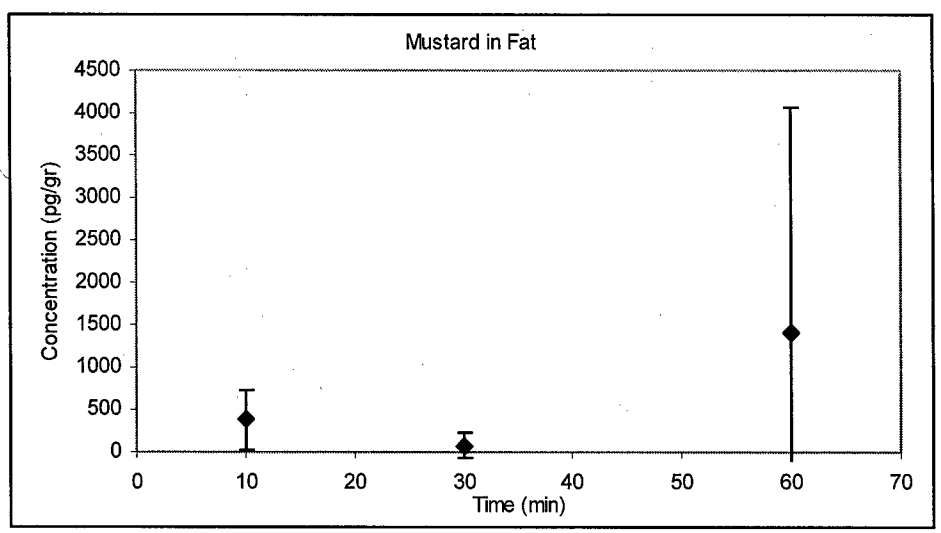

Figure 15. Concentration-time course of intact sulfur mustard ($pg.g^{-1}$, ± S.D.) in fat tissue of anesthetized, restrained marmosets at various time points after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

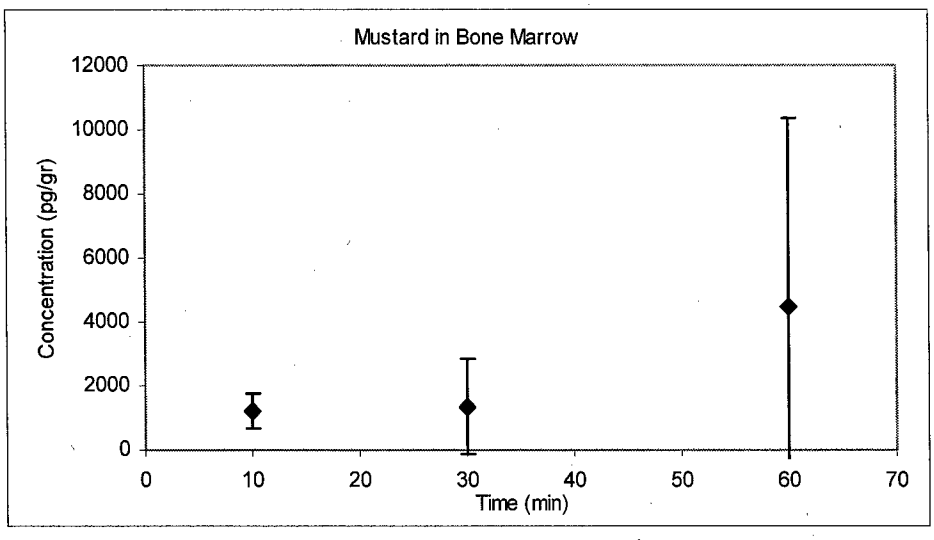

Figure 16. Concentration-time course of intact sulfur mustard ($pg.g^{-1}$, ± S.D.) in bone marrow of anesthetized, restrained marmosets at various time points after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

The highest concentrations of intact sulfur mustard are found in the bone marrow. The concentration is already high at 10 min after starting the exposure (ca. 1.2 $ng.g^{-1}$) and increases within the first hour to ca. 4.5 $ng.g^{-1}$. At 24 h after exposure, the concentration is below the detection limit (< ca. 10 $pg.g^{-1}$). In fact, in all tissues studied, the concentration of sulfur mustard at t=24 h is below the detection limit.
At t=10 min the concentration of sulfur mustard in the bone marrow is about 3-fold lower than that in the blood, but at t=60 min about 75 times higher.
In the liver the concentrations are already high at 10 min after starting the exposure (*ca.* 0.9 $ng.g^{-1}$), and decrease slowly with time.
In fat tissue the concentration builds up in the first hour after the exposure.
The concentrations in the spleen and lung are very low, and much lower than those in blood at the corresponding time points.

III.4.4. Toxicokinetics of 7-HETE-gua in blood and various tissues of the anesthetized marmoset at various time points after 5-min nose-only exposure to 160 mg.m-3, which corresponds with 1 LCt50 (96-h) in the hairless guinea pig (T.O. 6+8)

The concentrations of 7-HETE-gua in various marmoset tissues at various time-points after ending the nose-only exposure to sulfur mustard are presented in Tables 17 and 18. The concentration-time courses are shown in Figures 17 and 18.

Table 17. Concentrations (number of adducts per 10^7 nucleotides) of 7-HETE-gua in individual marmosets 5-min nose-only exposed to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

	T=10 Min				T = 30 Min					
Tissue	M008	M009	M010	M014	M015	M016	M018	M019	M020	M021
Nasal mucosa	20.0	7.7	1.5	23.0	8.4	12.5	11.8	1.3	28.8	3.7
Nasopharynx	17.2	25.8	4.9	19.8	5.6	6.7	8.7	2.2	7.1	3.2
Larynx	11.9	12.3	1.1	16.6	3.6	3.2	10.9	0.34	3.5	0.16
Trachea	9.8	17.4	0.96	21.7	6.1	10.6	15.4	0.64	6.5	5.4
Carina	21.0	12.4	1.8	17.0	1.8	3.6	16.0	2.5	3.9	2.7
Lung	0.02	0.04	0	0.29	0.12	0.15	0.11	0.07	0.39	0.3
Liver	0	0	0	0	0	0	0	0	0	0
Spleen	0.01	0	0	0	0	0	0.12	0.04	0	0
Bone marrow	0.04	0	0	0	0	n.m.	0.005	0.02	0	0.13
Small intestine	0.01	0	0	0.06	0	0	0.01	0.1	0	0.4
Blood	0.01	0.03	n.m.	n.m.	0.03	0.06	0.01	0.07	0.19	0.04

n.m. = not measured

	T=60 Min						120	24 H			
Tissue	M002	M003	M004	M005	M006	M007	M001	M011	M012	M013	M022
Nasal mucosa	17.0	16.9	15.8	12.6	23.4	23.9	18.6	4.2	11.2	22.6	1.5
Nasopharynx	19.7	9.5	17.8	3.0	14.9	9.1	12.2	3.7	6.6	10.0	4.4
Larynx	11.3	0.57	2.7	4.8	15.2	1.6	18.6	1.2	4.1	6.2	0
Trachea	18.2	4.4	15.8	9.1	12.5	3.2	17.9	2.8	6.5	11.8	0.49
Carina	17.9	1.1	9.3	7.2	8.5	8.2	17.8	2.1	4.5	8.5	0.55
Lung	0.6	0.06	0.17	0.07	0.06	0.13	0.19	0	0.09	0.03	0
Liver	0.02	0	0	0	0	0	0.03	0	0	0	4.7
Spleen	0.13	0	0.1	0.01	0.04	0	0.14	0.01	0	0	0
Bone marrow	0.07	0	n.m.	0	0	0	0.08	0	0.03	0	0
Small intestine	0.06	0	0	0	0.02	0.02	0.06	0	0.03	0	0
Blood	0.18	0.01	0.01	0.05	0.1	0.02	n.m.	0.23	0.08	n.m.	0.02

n.m. = not measured

Table 18. Mean concentrations of 7-HETE-gua ± SEM (number of adducts per 10^7 nucleotides, n=4 unless stated otherwise) in tissues of marmosets at various time points after the start of a 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard in air.

Tissue	T = 10 min	T = 30 min[a]	T = 60 min[a]	T = 120 min[b]	T= 24 h
Nasal mucosa	13 ± 5	11 ± 4	18 ± 2	19	9.8 ± 4.7
Nasopharynx	17 ± 4	5.6 ± 1.0	12 ± 3	12	6.2 ± 1.4
Larynx	10 ± 3	3.6 ± 1.6	6.0 ± 2.4	19	2.9 ± 1.4
Trachea	12 ± 5	7.4 ± 2.0	10 ± 2	18	5.4 ± 2.4
Carina	13 ± 4	5.1 ± 2.2	8.7 ± 2.2	18	3.9 ± 1.7
Lung	0.09 ± 0.07	0.19 ± 0.05	0.18 ± 0.09	0.19	0.03 ± 0.02
Liver	0 ± 0	0 ± 0	0 ± 0	0.03	0 ± 0
Spleen	0 ± 0	0.03 ± 0.02	0.05 ± 0.02	0.14	0 ± 0
Bone marrow	0.01 ± 0.01	0.03 ± 0.03	0.01 ± 0.01	0.08	0.01 ± 0.01
Small intestine	11 ± 6	0.09 ±0.07	0.02 ± 0.01	0.06	0.01 ± 0.01
Blood	0.02 ± 0.01	0.07 ± 0.03	0.06 ± 0.03	n.m.	0.11 ± 0.05

[a] n=6

[b] n=1

n.m. = not measured

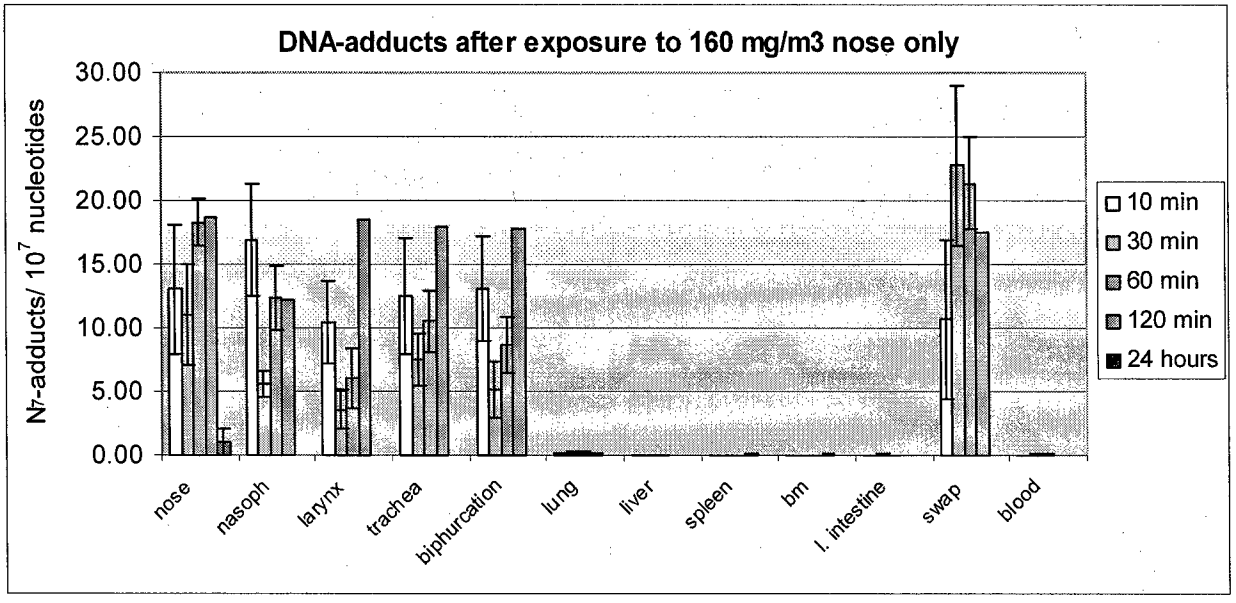

Figure 17. Mean concentration-time course of N7-HETE-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± SEM, in various tissues of marmosets 5-min nose-only exposed to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

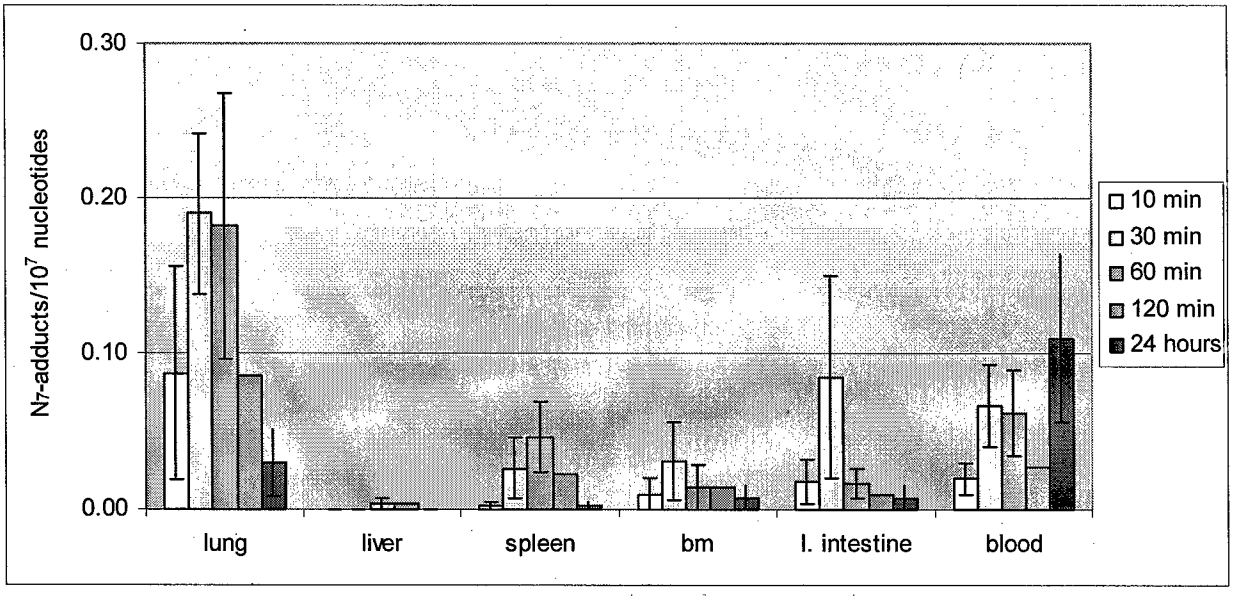

Figure 18. Mean concentration-time course of N7-HETE-gua, expressed as number of adducts per 10^7 nucleotides in DNA ± SEM, in various tissues of marmosets 5-min nose-only exposed to 160 $mg.m^{-3}$ sulfur mustard vapor in air (expanded view from Figure 17).

The highest adduct concentrations are found in the upper airways from the nasal mucosa down to the bifocation (carina). The concentrations in the lung are very low but higher than those in spleen, bone marrow, small intestine and blood. No adducts were found in liver, except at t=120 min in animal M001.
Due to the large standard deviations no significant differences can be discriminated between the values for the various tissueds and time points.

The adduct concentrations in the respiratory tract appear to reach their highest values already at t=10 min, after which they decrease. At t=24 h the adduct levels are 1.5-3-fold lower than at t=10 min. In particular the adduct level in the nasal mucosa remains relatively high.
The levels reported for t=120 min are higher than those at the other time-points, but since these numbers are obtained from only one animal it is not justified to base conclusions on these values.

III.4.5. Toxicokinetics of the terminal N-valine adduct of sulfur mustard to hemoglobin of the anesthetized marmoset at various time points after 5-min nose-only exposure to 160 mg.m^{-3}, which corresponds with 1 LCt50 (96-h) in the hairless guinea pig (T.O. 7)

The terminal N-valine adduct of sulfur mustard to hemoglobin (HETE-val) was measured in blood samples obtained from the experiments performed to determine the toxicokinetics of sulfur mustard in the blood of the marmoset (see paragraph III.4.2) and in tissues (see paragraph III.4.3).HETE-val was measured in these blood samples as described in paragraph II.4. The results are presented in Tables 19 and 20 and in Figure 19.

Table 19. Concentrations of HETE-val (µM) measured in blood of individual marmosets at various time points after 5-min nose-only exposure to 160 mg.m-3 sulfur mustard vapor in air.

Time (min)	M 001 Concentration HETE-Val (µM)	M 002 Concentration HETE-Val (µM)	M 003 Concentration HETE-Val (µM)	M 011 Concentration HETE-Val (µM)	M 012 Concentration HETE-Val (µM)	M 013 Concentration HETE-Val (µM)
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
30		0.25	n.d.			
60	n.d.	0.31	0.11			
120	0.11					
24 h				0.05	0.04	0.16

Time (min)	M 004 Concentration HETE-Val (µM)	M 005 Concentration HETE-Val (µM)	M 006 Concentration HETE-Val (µM)	M 007 Concentration HETE-Val (µM)	M 008 Concentration HETE-Val (µM)	M 014 Concentration HETE-Val (µM
0	n.d.	f.a.	n.d.	n.d.	n.d.	n.d.
10					0.07	0.10
30	0.30	0.08	0.11	0.0		
60	0.24	0.10	0.11	0.07		
24 h						

Time (min)	M 015 Concentration HETE-Val (µM)	M 016 Concentration HETE-Val (µM)	M 018 Concentration HETE-Val (µM)	M 020 Concentration HETE-Val (µM)	M 021 Concentration HETE-Val (µM)	M 022 Concentration HETE-Val (µM
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10						
30	f.a.	0.0	0.11	0.0	0.0	
60						
24 h						0.0

n.d = not detectable (< *ca.* 0.2 µM); f.a. = failed analysis

Table 20. Mean concentrations of HETE-val (μM) measured in blood of marmosets at various time points after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

Time (min)	Concentration HETE-Val (μM)	S.D. (μM)
0	0	0
10	0.07[a]	0.10
30	0.10[b]	0.12
60	0.13[c]	0.10
120	0.11[d]	0.0
24 h	0.07[e]	0.06

Average concentrations were calculated by interpreting n.d. as zero
[a] n=2
b n=11
c n=7
[d] n=1
[e] n=4

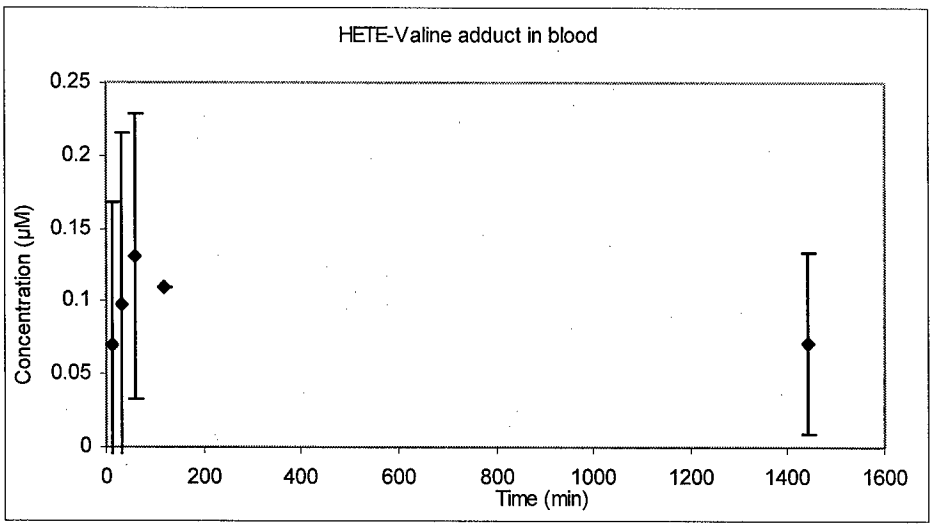

Figure 19. Mean concentration-time course of HETE-val (μM) in blood of marmosets at various time points after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

The mean concentration of HETE-val in blood builds up to *ca.* 0.13 μM in the first 60 min after starting the exposure to sulfur mustard vapor in air, and then seems to level off at t=120 min (n=1). At t=24 h the concentration is somewhat lower, but still easily detectable.

IV. DISCUSSION

Time course of distribution of N7-HETE-gua in the respiratory tract of the hairless guinea pig
After nose-only exposure to 0.3 or 1 LCt50 of sulfur mustard vapor in air, N7-HETE-gua levels build up in the respiratory tract of the hairless guinea pig. Subsequently, the adduct levels decrease, which is most likely due to the tendency of the body to repair DNA-damage.
After exposure to 1 LCt50, the concentration of adducts is more or less proportionally higher than after exposure to 0.3 LCt50, indicating that the binding sites for sulfur mustard to DNA are not saturated after exposure to 0.3 LCt50 of the toxicant.
There are some differences in the distribution of N7-HETE-gua within the respiratory tract between the two doses. After exposure to 0.3 LCt50 the highest adduct levels are found in the nasopharynx and trachea whereas no adducts are found in the lung. However, exposure to 1 LCt50 leads to almost equally high adduct levels in the nasal mucosa, nasopharynx, larynx, trachea and carina, whereas low levels are measured in the lung.

Inhalation toxicokinetics of sulfur mustard in the marmoset
With the improved gas chromatographic method, sulfur mustard could easily be measured in blood during and after 5-min nose-only exposure of anesthetized and restrained marmosets to 160 $mg.m^{-3}$ sulfur mustard vapor in air, which corresponds with 1 LCt50 in the hairless guinea pig. Previously we have measured the intravenous toxicokinetics of a dose corresponding with 1 LD50 in the hairless guinea pig (Langenberg *et al.* 1997).
Interestingly, the terminal half-life of 280 min as calculated for the inhalation toxicokinetics matches that of the intravenous toxicokinetics (270 min).
The area under the curve for the respiratory route is *ca.* 740 $ng.min.ml^{-1}$, which is about 20 times lower than after i.v. administration of 1 LD50 (14,450 $ng.min.ml^{-1}$). This seems a considerable difference, but it has to be realized that the internal doses are quite different. In the intravenous toxicokinetic study, *ca.* 3.3 mg of sulfur mustard were administered to a 400-g marmoset (corresponding with 8.2 $mg.kg^{-1} \equiv 1$ hairless guinea pig LD50). Assuming a respiratory minute volume of 100 $ml.min^{-1}$, a marmoset would inhale 0.4 mg of sulfur mustard in 5 min (external dose) when exposed to 800 $mg.min.m^{-3}$. Assuming that the retained fraction of the inhaled dose is 50 % would then lead to an internal dose of 0.2 mg, which is a factor 16.5 lower than the intravenous dose. In view of the fact that the sulfur mustard reacts in the respiratory tract a 20-fold difference is not unrealistic. Obviously this is just a rough calculation with a lot of assumptions. Nevertheless, the AUC-values for the two different routes seem to be reasonably comparable when corrected for the internal dose.

The most striking observations with respect to the time-course of the tissue distribution of intact sulfur mustard in the marmoset after nose-only exposure to this toxicant are the very low concentrations in lung and the very high concentrations in bone marrow. The first observation seems odd, since reasonably high levels of sulfur mustard are found in blood, and the whole blood volume passes through the lungs. Interestingly, only very low levels of 7-HETE-gua are found in the lung, which is in agreement with the measured low concentrations of intact sulfur mustard in the lung.
The DNA-adduct levels in bone marrow are very low, which is unexpected in view of the high concentrations of intact sulfur mustard found in this tissue.
After intravenous administration of 1 LD50 sulfur mustard to the marmoset, the adduct concentrations in lung and bone marrow were also relatively low, i.e., *ca.* 5 adducts per 10^7 nucleotides at 3 h after administration of the toxicant. The adduct levels in the lung of the marmoset appeared to be 10-fold lower than in the hairless guinea pig after intravenous administration of the same dose of sulfur mustard (8.2 $mg.kg^{-1}$), whereas the adduct level in the bone marrow of the marmoset was only 2-fold lower than that in the hairless guinea pig (Langenberg *et al.* 1997). Unfortunately, the concentrations of intact sulfur mustard in the tissues were not measured when studying the intravenous toxicokinetics of sulfur mustard in blood and N7-HETE-gua in

blood and tissues of the marmoset. This is a pity because now we cannot quantify the relationship between the concentration of sulfur mustard in blood, the concentration in tissues and the resulting concentrations of N7-HETE-gua in the tissues. On the basis of the concentration-time course of sulfur mustard in blood after intravenous administration, we hypothesized that apparently in the marmoset less partitioning of sulfur mustard from the blood to the tissues occurs (Langenberg *et al.* 1997). This hypothesis is still awaiting falsification.
Measurement of the concentration-time course of the adduct of sulfur mustard to hemoglobin, HETE-val, was intended to serve as an indicator for the toxicologically relevant concentration of sulfur mustard in blood. Unfortunately, due to reduction of the budget and thus the statement of work for this contract, the technical objective in which in a controlled experiment the concentrations of intact sulfur mustard would be linked to those of HETE-val was skipped. Concentrations of HETE-val were measured in blood samples of marmosets at various time points after starting the nose-only exposure to sulfur mustard vapor is air. Due to the high standard deviation of the measured values it is not possible to derive a minimum toxicologically relevant concentration of sulfur mustard from these results.

The inhalation toxicokinetics in the marmoset differ considerably from those in the hairless guinea pig (Langenberg *et al.* 1998). A 5-min nose-only exposure of hairless guinea pigs to 160 $mg.m^{-3}$ did not lead to measurable concentrations of sulfur mustard in blood. In agreement herewith, low concentrations of N7-HETE-gua were detectable in blood and lung tissue. The exposure dose had to be increased to 3 LCt50 in order to be able to measure sulfur mustard in the blood of the hairless guinea pig, and even then the concentrations were low: 1.8 $ng.ml^{-1}$ maximum. In the marmoset however, exposure to 1 hairless guinea pig LCt50 leads to concentrations of sulfur mustard in blood up to 30 $ng.ml^{-1}$. This observation seems to support our hypothesis that, due to the lower complexity of the upper airways, the marmoset is a more suitable model for man when studying the respiratory toxicology of sulfur mustard than the (hairless) guinea pig.

V. KEY RESEARCH ACCOMPLISHMENTS

1. Within a time period of 24 h after 5-min nose-only exposure of hairless guinea pigs to 48 $mg.m^{-3}$ sulfur mustard vapor in air (corresponding with 0.3 LCt50 (96-h)) the number of adducts in the respiratory tract increased and subsequently decreased within a 24-h time period, the latter most likely due to repair of the damage to DNA.

2. After nose-only exposure of hairless guinea pigs to 0.3 LCt50 of sulfur mustard, the highest number of DNA-adducts were found in the nasopharynx, followed by the larynx, trachea and the carina. No adducts were found in the lungs.

3. Within a time period of 24 h after 5-min nose-only exposure of hairless guinea pigs to 160 $mg.m^{-3}$ sulfur mustard vapor in air (corresponding with 1 LCt50 (96-h)) the number of adducts in the respiratory tract increased and subsequently decreased, the latter most likely due to repair of the damage to DNA.

4. After nose-only exposure of hairless guinea pigs to 1 LCt50 of sulfur mustard, the highest levels of DNA-adducts were found in the nasal mucosa, nasopharynx, larynx, trachea and carina. Low levels of DNA-adducts were found in the lungs.

5. The DNA-adduct levels in the upper airways appear to be reasonably proportional with the exposure dose.

6. A 'chair' was developed for fixation of marmosets in an upright position, in order to enable nose-only exposure of this species to toxicant vapor in air. In this way, the animals could be exposed in a natural posture.

7. The sensitivity of the gas chromatographic/mass-spectrometric method for measuring intact sulfur mustard in biological samples was improved by enhancing the selectivity of the chromatographic separation between the agent and the matrix by means of two-dimensional chromatography.

8. Upon 5-min nose-only exposure of marmosets to 160 $mg.m^{-3}$ sulfur mustard vapor in air, intact agent could be measured in blood during and up to at least 55 min after ending the exposure. The maximum concentration of sulfur mustard in blood was observed at the moment of ending the nose-only exposure and was *ca.* 30 $ng.ml^{-1}$ of blood.

9. Since intact sulfur mustard could only be measured in blood of hairless guinea pigs after nose-only exposure to a 3-fold higher dose (2,400 $mg.min.m^{-3}$), with a maximum concentration of *ca.* 1.8 $ng.ml^{-1}$ of blood, there is a marked difference between the inhalation toxicokinetics of sulfur mustard in the hairless guinea pig and in the marmoset.

10. The highest concentrations of sulfur mustard after a 5-min nose-only exposure of marmosets to 160 $mg.min.m^{-3}$ sulfur mustard vapor in air were found in the bone-marrow, liver and fat tissue. Very low concentrations were found in lung tissue.

11. The concentrations of adduct of sulfur mustard to DNA (N7-HETE-gua) in the marmoset following 5-min nose-only exposure to 160 $mg.m^{-3}$ were much higher in the upper airways than in the tissues studied. Very low adduct levels were found in the lung, which is in agreement with the low level of intact sulfur mustard measured in this tissue. Very low adduct levels were also

found in the bone marrow, which is unexpected in view of the high concentrations of intact agent found in this tissue.

12. Following 5-min nose-only exposure of marmosets to 160 $mg.m^{-3}$ sulfur mustard vapor in air concentrations of the adduct to the N-terminal valine of hemoglobin (HETE-val) were found of *ca.* 0.1 μM. HETE-val might be a marker for derivation of a minimum toxicologically relevant concentration of sulfur mustard in blood.

13. On the basis of the results obtained in this study, the marmoset seems to be a better model for man with respect to the toxicology of inhaled sulfur mustard vapor than the hairless guinea pig.

VI. REPORTABLE OUTCOMES

Publications

TRAP, H.C, KUIJPERS, W.C., DEGENHARDT, C.E.A.M., OOSTDIJK, J.P., MARS-GROENENDIJK, R.H., BIKKER, F.J., VAN DER SCHANS, G.P., BENSCHOP, H.P. AND LANGENBERG, J.P. (2005) Inhalation toxicokinetics of sulfur mustard in the marmoset. Proceedings of NATO TG-004, May 2005, Hradec Kralove, CZ.

Presentations

TRAP, H.C, KUIJPERS, W.C., DEGENHARDT, C.E.A.M., OOSTDIJK, J.P., MARS-GROENENDIJK, R.H., BIKKER, F.J., VAN DER SCHANS, G.P., BENSCHOP, H.P. AND LANGENBERG, J.P. (2005) Inhalation toxicokinetics of sulfur mustard in the marmoset. Oral presentation at NATO TG-004, May 2005, Hradec Kralove, CZ. (Presented by Dr. Herman P.M. van Helden).

VII. CONCLUSIONS

The time course of the distribution of DNA-adducts of sulfur mustard (N7-HETE-gua) in the respiratory tract of the hairless guinea pigs was studied for 5-min nose-only exposure to doses corresponding with 0.3 and 1 LCt50. After exposure to 0.3 LCt50 the number of adducts in the respiratory tract increased and subsequently decreased within 24 h. The highest number of DNA-adducts was found in the nasopharynx, followed by the larynx, trachea and the carina. No adducts were found in the lungs.
Within a time period of 24 h after exposure 1 LCt50 the number of adducts in the respiratory tract also increased and subsequently decreased. In this case approximately equally high numbers of DNA-adducts were found in the nasal mucosa, nasopharynx, larynx, trachea and carina. Low levels of DNA-adducts were found in the lungs. The DNA-adduct levels in the upper airways appeared to be reasonably proportional with the exposure dose.

Next, the inhalation toxicokinetics of sulfur mustard and its adducts to DNA and hemoglobin were studied in marmosets. For this purpose a 'chair' was developed for fixation of marmosets in an upright position, in order to enable nose-only exposure of this species to toxicant vapor in air in a natural posture.
The selectivity of the previously developed gas chromatographic/mass-spectrometric method for measuring intact sulfur mustard in biological samples appeared to be insufficient to study the toxicokinetics of sulfur mustard in blood of the marmoset. The selectivity was improved by performing two-dimensional chromatography.
Upon 5-min nose-only exposure of marmosets to 160 $mg.m^{-3}$ sulfur mustard vapor in air, intact agent could be measured in blood during exposure and up to at least 55 min after ending the exposure. The maximum concentration of sulfur mustard in blood was observed at the moment of ending the nose-only exposure and was *ca.* 30 $ng.ml^{-1}$ of blood. These results are in sharp contrast with those obtained previously in hairless guinea pigs, where the exposure dose had to be increased 3-fold before sulfur mustard appeared in the blood, albeit that the maximum concentration was only *ca.* 1.8 $ng.ml^{-1}$ of blood. Obviously, there is a marked difference between the inhalation toxicokinetics of sulfur mustard in the hairless guinea pig and in the marmoset, most likely due to differences in upper airway complexity.
Interestingly, the values for the terminal half-life of sulfur mustard and area-under-the-curve (when corrected for the dose difference) calculated for nose-only exposure of marmosets to sulfur mustard vapor in air corresponded very well with those previously calculated for intravenous administration
The highest concentrations of sulfur mustard after a 5-min nose-only exposure of marmosets to 160 $mg.min.m^{-3}$ sulfur mustard vapor in air were found in the bone-marrow, liver and fat tissue. Very low concentrations were found in lung tissue, which is a puzzling observation, since the whole blood volume passes through the lungs.
The concentrations of the adduct of sulfur mustard to DNA (N7-HETE-gua) in the marmoset following 5-min nose-only exposure to 160 $mg.m^{-3}$ were much higher in the upper airways than in the tissues studied. Very low adduct levels were found in the lung, which is in agreement with the low level of intact sulfur mustard measured in this tissue. Very low adduct levels were also found in the bone marrow, which is unexpected in view of the high concentrations of intact agent found in this tissue.
Following 5-min nose-only exposure of marmosets to 160 $mg.m^{-3}$ sulfur mustard vapor in air concentrations of the adduct to the N-terminal valine of hemoglobin (HETE-val) were found of *ca.* 0.1 µM. HETE-val might be a marker for derivation of a minimum toxicologically relevant concentration of sulfur mustard in blood.

On the basis of the results obtained in this study, the marmoset seems to be a better model for man with respect to the toxicology of inhaled sulfur mustard vapor than the hairless guinea pig.

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IX. LIST OF PERSONNEL RECEIVING PAY UNDER THIS COOPERATIVE AGREEMENT

Dr. Henk P. Benschop
Dr. Floris J. Bikker
Mrs. Carla E.A.M. Degenhardt-Langelaan
Mr. Alex Fidder
Ms. Marjan J. Jongsma
Ms. Wendy Kaman
Mr. Willem C. Kuijpers
Dr. Jan P. Langenberg
Mrs. Roos H. Mars-Groenendijk
Mr. John P. Oostdijk
Dr. Govert P. van der Schans
Mr. Henk C. Trap

ANNEX A. REDUCED STATEMENT OF WORK/TECHNICAL OBJECTIVES FOR COOPERATIVE AGREEMENT DAMD17-03-1-0613 (VERSION DECEMBER 3, 2004)

The goal set for the proposed research is:

Comparison of the inhalation toxicokinetics of sulfur mustard and its DNA-adducts in the hairless guinea pig and a species more relevant for man, i.e., the marmoset monkey.

Technical Objectives

Toxicokinetic studies on respiratory exposed animals

1. Determination of the concentration of N7-HETE-gua in DNA from samples of the mucosa of various sites of the respiratory tract (nasal mucosa, nasopharynx, larynx, trachea, carina, and lung) in groups of 4 hairless guinea pigs at 10 and 30 min, and at 1, 2 and 24 h after ending a 5-min nose-only exposure to sulfur mustard vapor in air with a concentration of 160 $mg.m^{-3}$, which corresponds with 1 LCt50 (96-h).

2. Determination of the concentration of N7-HETE-gua in DNA from samples of the mucosa of various sites of the respiratory tract (nasal mucosa, nasopharynx, larynx, trachea, carina, and lung) in groups of 4 hairless guinea pigs at 10 and 30 min, and 1, 2, 4 and 24 h after ending a 5-min nose-only exposure to sulfur mustard vapor in air with a concentration of 48 $mg.m^{-3}$, which corresponds with 0.3 LCt50 (96-h)

3. The apparatus designed within the context of Cooperative Agreement DAMD17-94-V-4009 for nose-only exposure of hairless guinea pigs to sulfur mustard vapor in air and used in the experiments as described in items 1 and 2 will be modified to allow the use of marmosets as an animal model.

4. Determination of the time course of the concentration of sulfur mustard in blood during and after a 5-min nose-only exposure of anesthetized, restrained marmosets to sulfur mustard vapor in air with a concentration of 160 $mg.m^{-3}$. Such an exposure corresponds with 1 LCt50 (96-h) in the hairless guinea pig.
   4.1 For this purpose the procedure for analysis of sulfur mustard in marmoset blood using gas chromatography with large volume injection and mass-selective detection will be optimized and validated, aiming at a detection limit of 10 pg of sulfur mustard per ml of marmoset blood.

5. Determination of the concentration of sulfur mustard in lung, liver, spleen, bone marrow and abdominal fat of 3 marmosets at halfway and at the end of the toxicokinetic experiment described under T.O. 4. Additional experiments will be performed to obtain tissue samples at 10 min after ending the 5-min nose-only exposure to sulfur mustard.

6. Determination of the concentration of N7-HETE-gua in DNA from blood, lung, liver, spleen, bone marrow and small intestine of 3 marmosets at 10 min after ending the 5-min nose-only exposure to sulfur mustard, as well as halfway and at the end of the toxicokinetic experiment described under T.O. 4. Additional experiments will be performed to obtain tissue samples at 24 h after ending the 5-min nose-only exposure to sulfur mustard.

7. Determination of the concentration of the terminal N-valine adduct of sulfur mustard to hemoglobin in the marmoset blood samples taken in the toxicokinetic experiment described under T.O. 4, and in the blood samples taken at 24 h after ending the 5-min nose-only exposure, described under T.O. 6.

8. Determination of the concentration of N7-HETE-gua in DNA from samples of the mucosa of various sites of the respiratory tract (nasal mucosa, nasopharynx, larynx, trachea, carina, and lung) of 3 marmosets at the end of the toxicokinetic experiment described under 4.

9. If a toxicokinetic profile of sulfur mustard in blood cannot be determined in two subsequent animal experiments under the conditions described under T.O. 4, marmosets will be nose-only exposed to sulfur mustard vapor in air with a concentration of 300 $mg.m^{-3}$ for 8 min. Such an exposure results in a Ct which is three times the LCt50-value for 5-min exposure in the hairless guinea pig. In that case, all experiments described under T.O. 4-8 will be performed with this higher exposure level.

Time Schedule

The technical objectives will be performed over a period of two years:
1st year: technical objectives 1 to 4 (partly)
2nd year: technical objectives 4 (remainder) and 4.1 to 9

ANNEX B STANDARD OPERATING PROCEDURE FOR DETERMINATION OF N7-HETE-GUA IN DNA

Materials

DNA isolation

Cell lysis solution	10 mM Tris, 1mM EDTA, 1% v/v SDS
DNA isolation kit	PureGene kit, Biozym
PBS (sterile)	Phosphate Buffered Saline (Dulbecco's)

Capped Eppendorf tubes (1.5 ml)
Gilson pipets (20, 200, 1000 µl) and tips
Eppendorf centrifuge, model 5417 C
Proteinase K
Rnase A
Isopropanol
70% Ethanol
Filterpaper
Water bath at 37 °C
Rotating wheel shaker
Incubator at 37 °C

Vibrator	Titertek (Flow)
0.1TE buffer	1 mM Tris-HCl, 0.1 mM Na2EDTA, pH 7.4
UV/VIS spectrometer	E.g., Lambda 40, Perkin Elmer, Breda, The Netherlands

DNA denaturation

Calf thymus DNA calibration samples	Calf thymus DNA exposed to 0, 2.5, 5, 10 and 20 nM sulfur mustard
TE buffer	10 mM Tris-HCl, 0.01 mM Na2EDTA, pH 7.4
MQ water	Purified tapwater, classification I, ISO 3696
Formamide	99%
Formaldehyde	36.5%

52 °C water bath
Gilsonpipets (20, 200, 1000 µl) and tips

Immunoslotblot assay

Nitrocellulose	Protran BA 79, nitrocellulose transfer membrane, 0.1 µm, Schleicher and Schuell
Blotting paper	Gel-blotting-paper GB 002 (0.8 mm), Schleicher and Schuell
Blotting device	Minifold 1 Dotblot manifold, Schleicher and Schuell

Vacuum pump
Glass vacuum flask
12-channel pipet and tips
Gilsonpipets (20, 200, 1000 µl) and tips
Flat forceps e.g. Millipore
Gloves

PBS (sterile)	Phosphate Buffered Saline (Dulbecco's)
MQ water	Purified water, classification I, ISO 3696
Milkpowder	ELK, skimmed milkpowder, less than 1% fat, Campina, Eindhoven, The Netherlands
1st Antibody, 2F8	Directed against against N7-HETE-Gua, culture supernatant, TNO-PML, Rijswijk, The Netherlands
2nd Antibody	Rabbit-anti-mouse-Ig-horse radish peroxidase, Dakopatts, Glostrup, Denmark

Enhanced Chemiluminescence

Blotting Detection System	Solution A and B, Boehringer Mannheim, Germany
Incubation boxes	
UV-gene cross-linker	E.g., GS Gene Linker UV chamber, Bio-Rad Laboratories, The Netherlands
Waterbath at 25 °C	
Luminometer	E.g., MicroBeta Trilux 6 detector system, Wallac, EG & G Berthold
Plastic sheets	For packing blots
Shaking plate	
Stopwatch	
Filter paper	

Procedure for immunoslotblot asssay to detect sulfur mustard adducts to DNA in respiratory tissue of the hairless guinea pig

Sampling

DNA from mucosal scrapings of the nasal cavity, nasopharynx, larynx, trachea and carina was isolated and treated as described below. Lung tissue was homogenized prior to DNA isolation.

DNA isolation

1. Mucosal scrapings/homogenized lung tissue are transferred into 1.5-ml Eppendorf tubes.
2. Add Cell lysis solution (300 µl), supplemented with Proteinase K (100µg/ml).
3. Incubate 16 h at 37 °C under continuous shaking.
4. Treat with RNase A (1.5 µl, 50 µg/ml) for 15 min at 37 °C, followed by cooling on ice for min 5 min.
5. Add Protein Precipitation Solution (100 µl), mix on a high speed vortex (20 s).
6. Cool on ice for 5 min.
7. Centrifuge at 14,000g for 10 min.
8. Transfer the supernatant to a tube containing isopropanol (300 µl) in order to precipitate the DNA, and centrifuge at 7,000 rpm (5,200g) for 5 min.
9. Wash the pellet with 70% ethanol (300 µl), centrifuge (7,000 rpm, 5 min), and dry on air for *ca.* 15 min.
10. Dissolve the pellet in 0.1TE buffer (50 or 100 µl depending on the size of the pellet) under continuous vibration overnight at room temperature. (Dissolution can be accomplished within 1 to 2 h when performed with fresh blood)
11. Determine DNA concentration by diluting the DNA solution (4 µl) 20-fold with 0.1TE buffer and measure A_{260} in a 1-cm quartz microcuvette in a UV/VIS spectrometer (1000 * A260 = DNA concentration in µg/ml of the undiluted solution). Measure also A_{280} as indication for the purity of the DNA solution. (The A_{260}/A_{280} ratio should be between 1.6 – 1.9)

DNA denaturation

1. Make up solutions (100 µl) with final concentrations of DNA (50 µg/ml), formamide (4.1%), and formaldehyde (0.1%) in 0.1TE buffer, incubate at 52 °C for 15 min, and cool rapidly on ice. Store at –20 °C (freezing of the samples at least once is essential). Treat the calf thymus DNA calibration samples in the same way.

Immunoslotblot procedure

1. Dilute the denatured DNA samples in PBS to a final concentration of 5 µg/ml (including the calf thymus DNA calibration samples)
2. Assemble the blotting manifold: connect with vacuum flask and place 2 pieces of blotting paper (wear gloves); make a nitrocellulose filter, cut in a 96-well format, wet (with water and PBS) and place it on the upper part of the manifold (without air bubbles); place the upper part on the other parts and fix the clamps. Switch on the vacuum pump.

3. Spot the DNA solution (200 µl) in duplicate. Do not use positions A12 and H1. (These positions are needed as markers for the positioning of the filter in the luminometer cassette.)
4. Wash each dotted sample with PBS (400 µl) by suction through the filter.
5. Take the nitrocellulose filter from the blotting manifold and dry on air for 10-15 min.
6. Cross-link the DNA to the filter by means of illumination with the UV-gene-cross-linker (50 mJ/cm^2).
7. Incubate the filter with blocking solution (about 50 ml, 5% milkpowder in PBS + 0.1% Tween 20) at room temperature for 30 min.
8. Wash three times with PBS + 0.1% Tween 20.
9. Incubate the filter with 1st antibody diluted 500-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, overnight at 4 °C under continuous shaking.
10. Wash 4 times with PBS + 0.1% Tween 20; the last three times for at least 15 min each.
11. Incubate the filter with 2nd antibody diluted 1000-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, for 2 h at room temperature under continuous shaking.
12. Wash 4 times with PBS + 0.1% Tween 20; the last three times for at least 15 min each.
13. Incubate solution A (of the Enhanced Chemiluminescence Blotting Detection System) in a waterbath at 25 °C until temperature equilibrium is reached. Mix solution B with solution A in a ratio 1:100 and preincubate the substrate solution for at least 30 min at 25 °C.
14. Remove free (wash) solution from the filter with filter paper, mark position A12 and H1 with ball point (not a felt pen!).
15. Place the filter in a closely fitting box, add 10 ml of substrate solution and incubate for 1 min.
16. Wrap the filter, straight from the substrate in plastic, without air bubbles. Press out liquid, transfer the filter in plastic into the luminometer cassette and place it in the luminometer.
17. Measure luminescence according to the required program. Collect data in a file and calculate for each sample the level of N7-HETE-Gua/10^7 nucleotides (in an Excel worksheet).

ANNEX C CONCENTRATION-TIME COURSE OF SULFUR MUSTARD IN BLOOD OF INDIVIDUAL MARMOSETS 5-MIN NOSE-ONLY EXPOSED TO 160 $mg.m^{-3}$ SULFUR MUSTARD VAPOR IN AIR

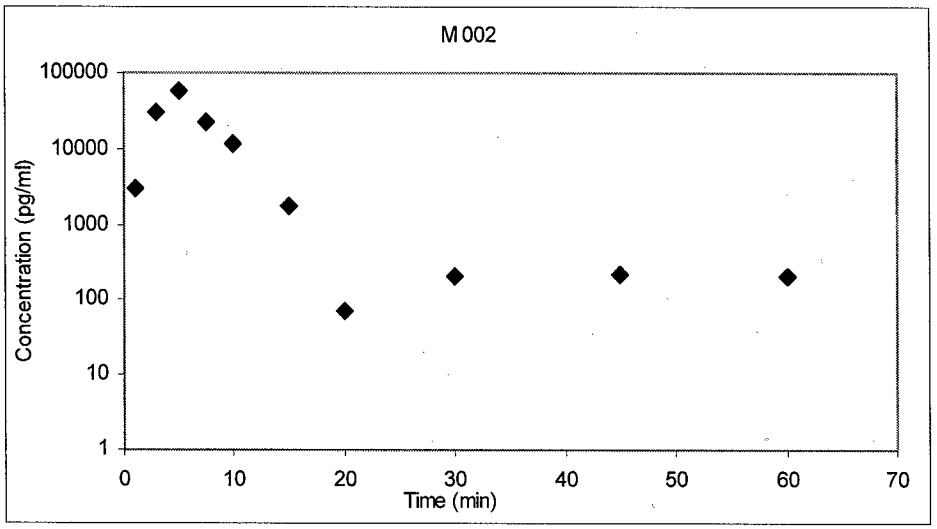

Figure 20. Concentration-time course of sulfur mustard in blood ($pg.ml^{-1}$) of marmoset M002 during and after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

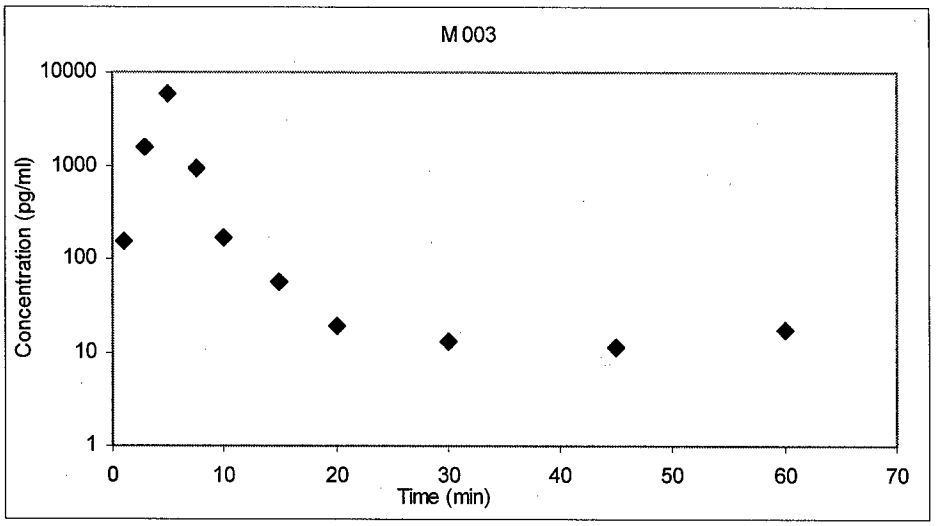

Figure 21. Concentration-time course of sulfur mustard in blood ($pg.ml^{-1}$) of marmoset M003 during and after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

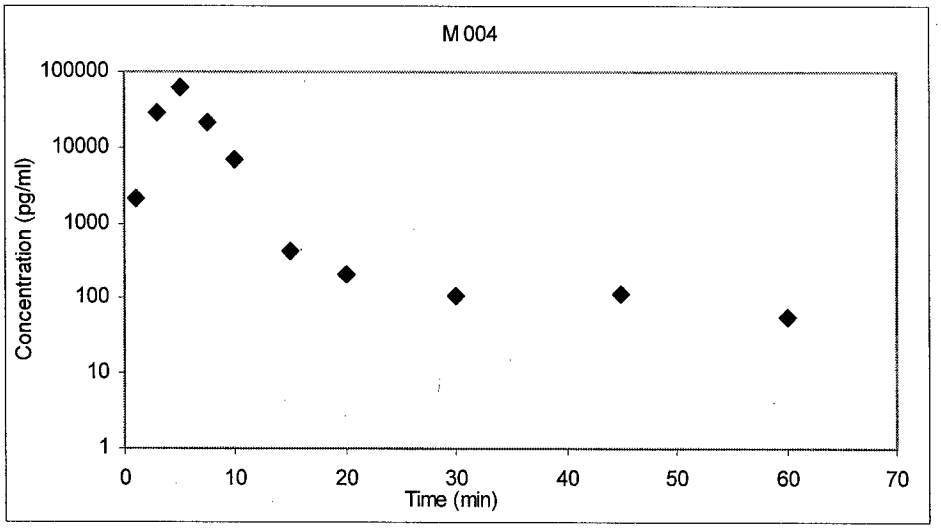

Figure 22. Concentration-time course of sulfur mustard in blood ($pg.ml^{-1}$) of marmoset M004 during and after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

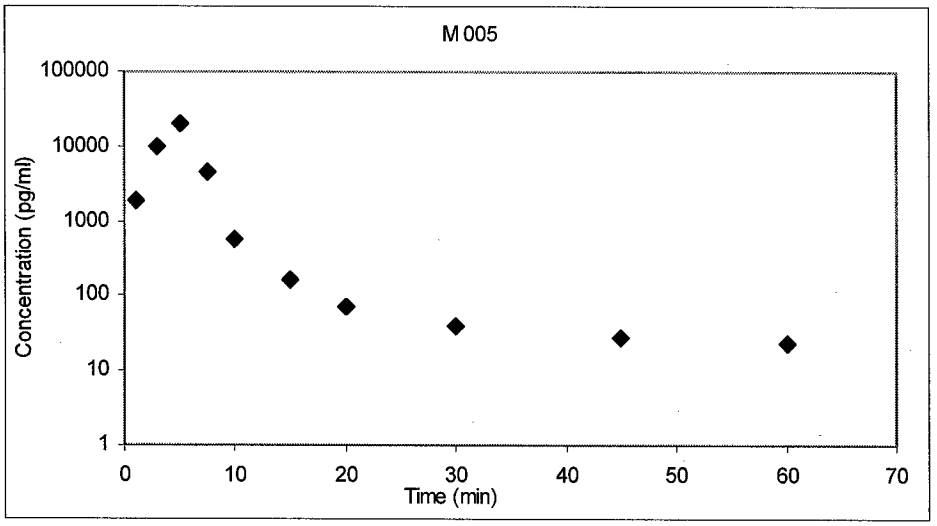

Figure 23. Concentration-time course of sulfur mustard in blood ($pg.ml^{-1}$) of marmoset M005 during and after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

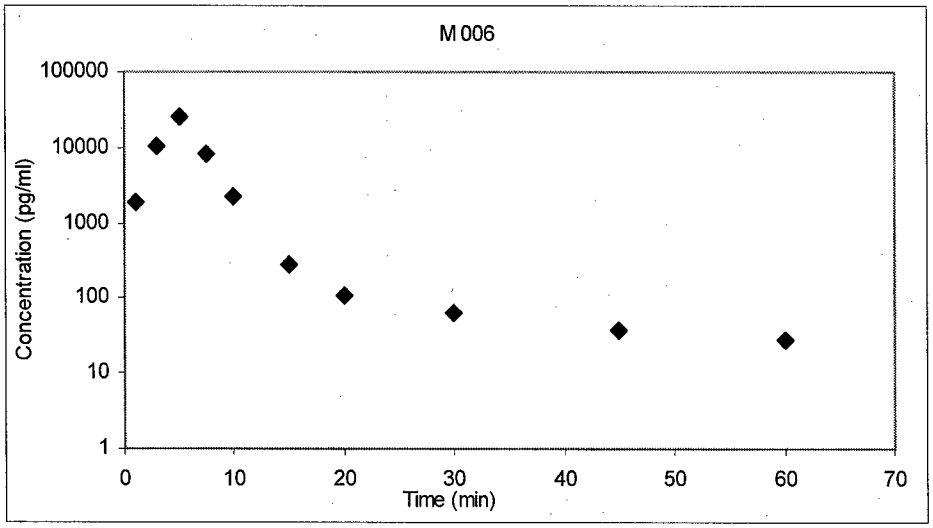

Figure 24. Concentration-time course of sulfur mustard in blood ($pg.ml^{-1}$) of marmoset M006 during and after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

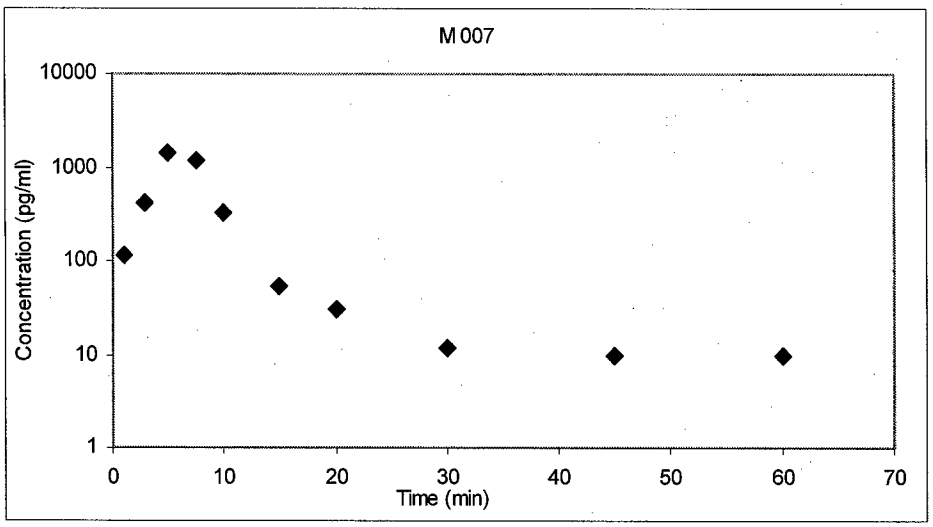

Figure 25. Concentration-time course of sulfur mustard in blood ($pg.ml^{-1}$) of marmoset M007 during and after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

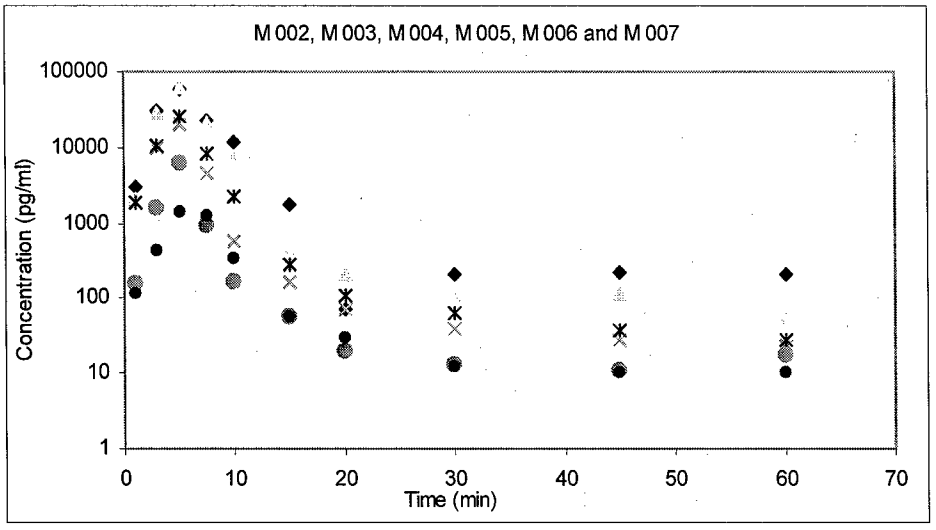

Figure 26. Overview of the concentration-time courses of sulfur mustard in blood ($pg.ml^{-1}$) of marmosets M002-M007 during and after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

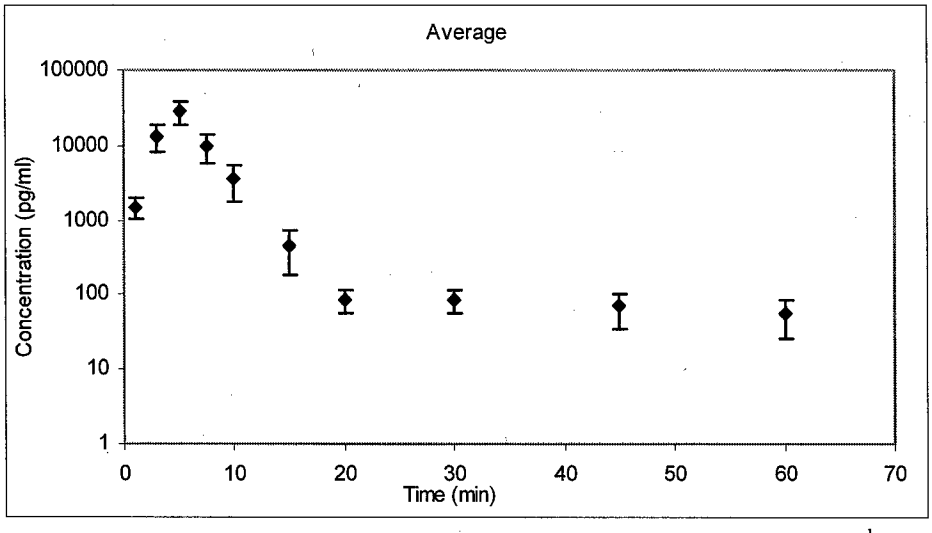

Figure 27. Mean concentration-time course with SEM of sulfur mustard in blood ($pg.ml^{-1}$) of marmosets M002-M007 during and after 5-min nose-only exposure to 160 $mg.m^{-3}$ sulfur mustard vapor in air.

ANNEX D ACRONYMS AND ABBREVIATIONS

A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
CI	Chemical ionization
Ct	Product of concentration and exposure time
CW	Chemical warfare
DNA	Deoxyribonucleic acid
ε	Molar extinction coefficient
ECD	Electron capture detector
EI	Electron impact
ELISA	Enzyme linked immunosorbent assay
FID	Flame ionization detector
FPD	Flame photometric detector
GC	Gas chromatography
ISB	Immunoslotblot
LC	Liquid chromatography
LCt50	Product of concentration and exposure time that produces 50 % mortality in a population
LD50	Dose that produces 50 % mortality in a population
LIF	Laser-induced fluorescence
MS	Mass spectrometer
MSD	Mass-spectrometric detector
MuSIC	Multiple Switching Intelligent Controller
N7-HETE-gua	N7-hydroxyethylthioethyl guanine
NBC	Nuclear, Biological, Chemical
NICI	Negative ion chemical ionization
PBS	Phosphate buffered saline
PML	Prins Maurits Laboratory
SIM	Selected ion mode
TDSA	Automated thermodesorption sampler
TIC	Total ion current
TNO	Netherlands' Organization for Applied Scientific Research
TSP	Topical skin protectant
USAMRICD	U.S. Army Medical Research Institute of Chemical Defense
USAMRMC	U.S. Army Medical Research and Materiel Command
UV	Ultraviolet